69 p.

N64 11103

CODE-1

NASA CR 2607

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NASA CR 52607

0166009

Aeronutronic, Newport Beach, Calif.

QUARTERLY ENGINEERING PROGRESS REPORT,

STUDY OF BASIC BIO-ELECTROCHEMISTRY

☒ OTS
☐ 2

Prepared for: National Aeronautics and Space Administration
Washington, D. C.

Under Contract: *(NASA Contract)* NASw-655)

Reporting Period: 20 June to 19 September 1963

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19 October 1963

(NASA CR-52607; U-2332)

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SUMMARY

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1. The survey of enzyme systems pertinent to the field of bioelectro-
    chemistry has continued with the testing of two sugar oxidases from
    different sources, a diaphorase system, arginase, and an amino acid
    decarboxylase, L-glutamic acid decarboxylase. None of these was
    found effective for current production at normal electrode potentials
    without use of carriers.

2. The bioelectrochemical activity of the D-amino acid oxidase trypto-
    phan system has been extensively characterized. A combination of
    electrochemical, spectrophotometric and chronopotentiometric tech-
    niques has demonstrated that the major source of current in this
    system is the electrode oxidation of indole pyruvic acid, one of the
    major products of the enzyme-substrate reaction. Further, it appears
    that the indole pyruvic acid exists in more than one tautomeric form
    in solution. One of these, the "enol" is oxidized at 0.12v, the
    other "keto" form at 0.6 to 0.63v. Little evidence exists for
    participation of any other reaction as a highly significant factor
    in current production with this system.

3. Work on the urea-urease system suggests that early results, demon-
    strating good current values at electrode potentials of 0.2 v. vs
    SCE, may have been due to impurities in the system (e.g. mecaptans).

4. Studies on the attachment of biological agents to electrode surfaces,
    and the characteristics of such attached agents have been started
    but are in a rather premature state for conclusions to be made.

5. A highly significant finding, from chronopotentiometric studies, has
    been that high electrode oxidation potentials may result in poisoning
    of the electrode with adsorbed oxidation products of the electro-
    active species.

AUTHOR

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SECTION 1

INTRODUCTION

As outlined in the first quarterly report, this program involves a study of the basic processes involved in the generation of electrical energy by biological systems. It is planned to be carried out in three phases. The first phase involves a survey of a number of classes of biological systems for the purpose of designating those of greatest electrochemical activity. This phase was substantially complete at the end of the first quarter, however, some additional systems were examined briefly during this report period. The details of this examination are given in a later section. None of the systems involved were found to possess electrical activity.

The second phase of the program is concerned with a detailed study of one or more biological systems found to be the most active in the generation of electrical energy. Some possible additional discretion in the selection of such systems based upon such factors as amenability to study, potential usefulness in waste disposal applications, etc., was anticipated to be desirable. Information obtained during the first quarter and reported in the previous quarterly report has indicated that the enzyme, D-amino acid oxidase, with tryptophane substrate was one such system. Another, based upon work reported from a number of different laboratories, is the urease-urea system. This latter is of particular

interest since the action of urease on urea is nominally hydrolysis to ammonia and CO_2 with no oxidation-reduction processes involved. The source of electrochemical activity is therefore a question of considerable scientific as well as practical concern. Available information indicates the activity to be greater than can be explained in terms of the electrochemical oxidation of ammonia.

Both of these systems have been studied during this report period, greatest emphasis being on the D-amino acid oxidase (DAO) - tryptophan system. The studies have been primarily by means of three experimental techniques.

The most powerful of these, namely chronopotentiometry, was discussed briefly in the last quarterly report. The underlying principles and the significance of the results obtainable by means of this technique were described therein. A second technique involves the spectrophotometric analysis of the products of the enzyme oxidation of amino acids and study of the correlation between the formation of these products and the generation of bio-electricity. The third method directed to study and identification of the products of the electrode reaction has been given preliminary attention only during this period. It depends upon separation and identification of the various constituents of the electrochemical cell electrolyte by means of paper electrophoresis.

The third phase of this program concerns the more practical problem of optimizing output from a given system through the attachment of organisms to the electrode and associated effects related to the diffusion of substituents through the attached film, as well as other aspects of electrode configuration. The reaction of biological systems in an electrochemical cell requires the assumption either of direct participation of the enzyme, or some other macromolecular structure such as the enzyme-substrate complex, in the electrode reaction, or an indirect participation in which the enzyme or biological catalysts participate

only in the production of an electrochemically reactive species from a previously inactive substrate. A number of modifications of each type of system including combinations of both may be visualized. Although distinctions between these two cases may be readily made in many systems, the situation in an intact organism may not be easily clarified without a full knowledge of the physical parameters of the electrode-organism system. Thus, it is essential to establish the relation between electrical output and variation in factors such as the proximity of enzyme to electrode, masking effect of protein upon collector surfaces, orientation of enzyme to electrode surface and possible electrical conductivity of biological material in serving as a direct bridge from reaction to electrode surface. As a first approach to this problem, it has been undertaken to determine the effect of parameters which would affect diffusional characteristics of electroactive material in the region of the electrode. This problem is discussed in more detail in Section 6 and some exploratory studies are described concerning the effect of varying enzyme concentrations in the region of the electrode through the use of a supporting agar matrix or by enclosing in semipermeable membranes.

SECTION 2

MATERIALS AND METHODS

2.1 MATERIALS

    a.   <u>Enzymes</u>

The crystalline enzymes, D-amino acid oxidase (DAO) and urease were prepared at Aeronutronic as previously described in the first quarterly report. DAO had an activity of approximately 306μ/mg (μl O_2 uptake/30min/mg) and urease an activity of 11 to 13 Sumner units/mg (Sumner unit, designated Su = 1 mg N ammonia released per 5 minutes). Commercial urease has also been used. Other commercial enzyme preparations were: Calbiochem, urease, 0.34 SU/mg; Mann Research Laboratory, diaphorase from <u>Clostridium kluyveri</u>, 974 u*/mg and crystalline (2x) rabbit muscle lactic dehydrogenase, 31.3 u/mg; Sigma Chemicals, Catalase, beef liver, 8250 u/mg; Worthington Bio-Chemical Co., L-arginase from beef liver, 20 u/mg, L-glutamic decarboxylase from <u>E. coli</u>, 0.6 μ/mg.

The sugar oxidases from <u>Iridophycus flaccidum</u> and from orange fruit were prepared at the University of California by published procedures (1,2).

    b.   <u>Chemicals</u>

D-amino acids (alanine, tryptophan, tyrosine and phenylalanine) were from Sigma Chemical Co. Indole-3-pyruvic acid came from K & K Chemical

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$*$ Where enzymatic unit is not defined, one unit is that activity producing 1 μ mole product per minute (standard enzyme unit).

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Company while sodium phenyl pyruvate and p-hydroxyphenyl pyruvic acid were from Calbiochem Corporation. Other materials were as listed in the previous report.

2.2 METHODS

Standard experimental techniques or these discussed in previous reports are described here. New techniques developed during this report period are presented under the related sections.

a. <u>Potentiostatic Studies of Cell Output</u>

Unless otherwise stated, electrical measurements were performed in the electrochemical cell illustrated in Figure 3 of the first quarterly report. Electrical output was measured at a regular electrode potential of 0.200 v. vs. SCE maintained by means of a manually controlled power supply as outlined in the previous report. In anaerobic experiments, the cell was de-aerated through the use of prepurified nitrogen, further treated by passing over heated copper. This was passed over the surface of the anolyte solution in the cell, with stirring by means of a magnetic stirrer. Experiments designated as aerobic were carried out with the cell open to the air.

A micro-electrochemical cell shown in Figure 1 has been fabricated for use in tracer experiments. It uses one tenth the volume of anolyte required by the regular cell. Stirring is accomplished by a vibrating electrode driven by an electric shaver motor. This cell was tested by comparison of its output with that of the regular cell under otherwise the same conditions, and was found to show essentially identical behavior.

b. <u>Chronopotentiometry Studies</u>

The chronopotentiometric apparatus used in this investigation has been described in the first quarterly report on this program. A slight modification in the circuitry was made by the introduction of a
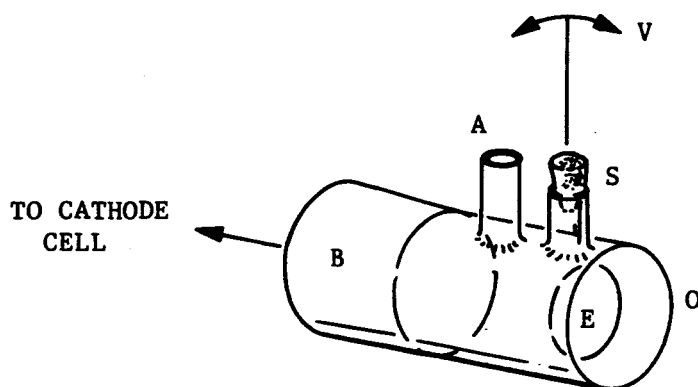
B - BODY OF CELL FROM   14/20 JOINT

E - PLATINUM ELECTRODE

A - OPENING FOR INSERTION OF LUGGEN CAPILLARY, NITROGEN
    FLOW CAPILLARY AND SYRINGES FOR ADDITION AND REMOVAL
    OF CONTENTS

V - ATTACHMENT TO ELECTRIC RAZOR MOTOR FOR VIBRATOR.
    AMPLITUDE VARIED FROM 0 TO 2 MM AT POINT OF ATTACH-
    MENT THROUGH USE OF POWERSTAT

S - MINIATURE RUBBER STOPPER ACTING AS FULCRUM FOR
    VIBRATING ELECTRODE

O - OPENING FOR ELECTRODE INSERTION, COVERED WITH
    PLASTIC CAP OR STOPPER

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FIGURE 1. MICRO-ELECTROCHEMICAL CELL

current reversing switch. The cell was of the same design as that employed in potentiostatic polarization experiments. (See Figure 3, p. 11 of first quarterly report.) The reference electrode (SCE) and the auxiliary electrode were connected to the electrolytic cell (test electrode compartment) through a fritted glass disc and a 3.0% agar plug saturated with potassium nitrate. The test electrode consisted of a smooth platinum disc (approximately $\frac{1}{4}$ inch in diameter) which was sealed to an end of a soft glass tubing. Electrical contact was made by means of a nickel wire welded to the platinum disc. This electrode was inserted in a larger diameter glass tubing which served as a mantle. The mantle extended approximately one inch below the surface of the solution in the cell. Anodic chrono-potentiometric studies were carried out with "oxidized", "reduced" and "clean" electrodes (defined below) in unstirred and stirred solutions. When measurements were made in unstirred solution the electrode was withdrawn approximately $\frac{1}{4}$ inch from the end of the glass mantle; in stirred solution the electrode was extended slightly beyond the end of the mantle. In anaerobic studies the solution was de-aerated prior to an experimental run and blanketed during the run with prepurified nitrogen. The preparation of D-tryptophan-D-amino acid oxidase solution involves special precautions to remove oxygen from the system. After the addition of required amount of D-tryptophan to the de-aerated buffer solution in the electrolytic cell the resulting solution was de-aerated further with nitrogen for 20 minutes. The amino acid oxidase was subjected repeatedly to vacuum and nitrogen atmosphere and then transferred to the cell with a hypodermic syringe. In all studies the temperature of the cell was maintained at $38^{o}C$.

"Oxidized" electrode refers to an electrode which was brought chronopotentiometrically to the potential of oxygen evolution. "Reduced" electrode refers to an oxidized electrode that was reduced chronopotentiometrically to the potential of hydrogen evolution. "Clean" electrode

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refers to an electrode which was treated in the following way: anodized and cathodized in 50% nitric acid solution, cathodized in H_2SO_4 (pH=0), rinsed with distilled water, wiped dry, placed in the sample solution and reduced chronopotentiometrically to a potential of hydrogen evolution. This treatment was found necessary to remove the oxidation products from the previous experimental run. The electrode was subjected to one or the other of these pretreatment procedures prior to the taking of each potentiogram.

    c.   <u>Other</u>

Spectrophotometric studies made use of either a Cary Model 14 or Beckman DK-2A recording spectrophotometer. Electrophoresis was performed with Research Specialties Co. equipment.

SECTION 3

SURVEY OF NEW ENZYME SYSTEMS

3.1 SUGAR OXIDASES

The glucose oxidase from <u>Penicillium</u> has been extensively studied in the electrochemical cell here and elsewhere. The sugar oxidases from <u>Iridophycus</u> <u>flaccidum</u> (1) (a red alga) and from oranges (2) have considerably different substrate and electron carrier specificities and might give different electrode reactions. In particular, the enzyme from oranges occurs in both a soluble and insoluble form (firmly bound to particulate material but fully active in suspension) and conceivably would lend itself to electrode attachment studies more readily than some soluble enzymes. None of these systems were sufficiently active in producing current to warrant further study.

3.2 LACTIC DEHYDROGENASE-DIAPHORASE-NAD SYSTEM

Diaphorase is a flavoprotein (3) which oxidizes reduced nicotinamide adenine dinucleotide ($NADH_2$). Although the latter cannot be considered as a likely fuel cell substrate, the large number of enzymes which use NAD as an electron carrier in the oxidation of simple substrates makes the diaphorase system attractive for study. In these experiments, the lactic acid-lactic dehydrogenase system was used to provide a continuous source of reduced NAD for reduction of the diaphorase. Once more, little activity was found.

3.3 ARGINASE

The seeming success of the urea-urease system in early studies made it desirable to check some related hydrolytic systems. Arginase, which hydrolyzes arginine to urea and ornithine, was tested for electrochemical activity and found inactive.

3.4 AMINO ACID DECARBOXYLASES

The amino acid decarboxylases are widely distributed in bacteria, one of the richest sources being _Escherichia coli_. They are generally formed in adaptive response to a specific amino acid and tend to be highly specific. The general reaction for these enzymes is $RCHNH_2COOH \rightarrow RCH_2NH_2 + CO_2$. Despite the specificity of individual enzymes, they all appear to function through the same mechanism, involving a prosthetic group (firmly attached to the enzyme) of pyridoxal phosphate.

The tests of L-glutamic decarboxylase showed no significant activity in the electrochemical cell.

SECTION 4

STUDIES ON THE AMINO ACID OXIDASE SYSTEM

4.1 GENERAL OBSERVATIONS

The characteristic reactions in this system are the following:

$$RCH_2NH_2COOH + En \longrightarrow RC(=NH)COOH \cdot EnH_2$$

$$\begin{pmatrix} \text{enzyme-substrate} \\ \text{complex} \end{pmatrix}$$

$$\xrightarrow{\text{anaerobic} + A} RC(N=H)COOH + H_2A + En$$

$$\xrightarrow{\text{aerobic} \quad O_2} RC(N=H)COOH + En + H_2O_2$$

$$RC(N=H)COOH + H_2O \longrightarrow R-CO-COOH + NH_3$$
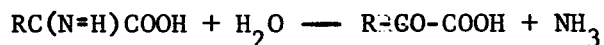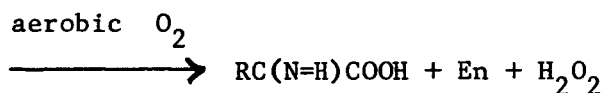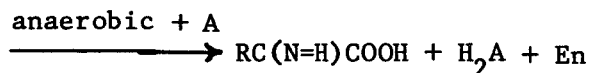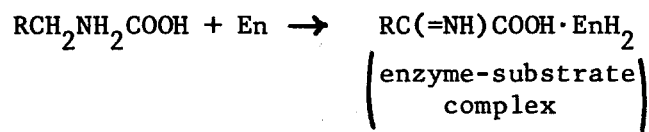
Information obtained the previous quarter and reported in the past quarterly report showed that significant currents could be obtained at relatively low electrode potentials from a system which included the enzyme D-Amino Acid Oxidase (DAO) acting upon certain aromatic amino acids under ostensibly anaerobic conditions.

Of particular interest was the high current obtained specifically with tryptophan. Figure 2 shows the magnitude of this current obtained under aerobic conditions as a function of time starting from first addition of the tryphophan and with the addition of successive increments of tryphophan thereafter. It is observed that in each case an initial transient

-11-
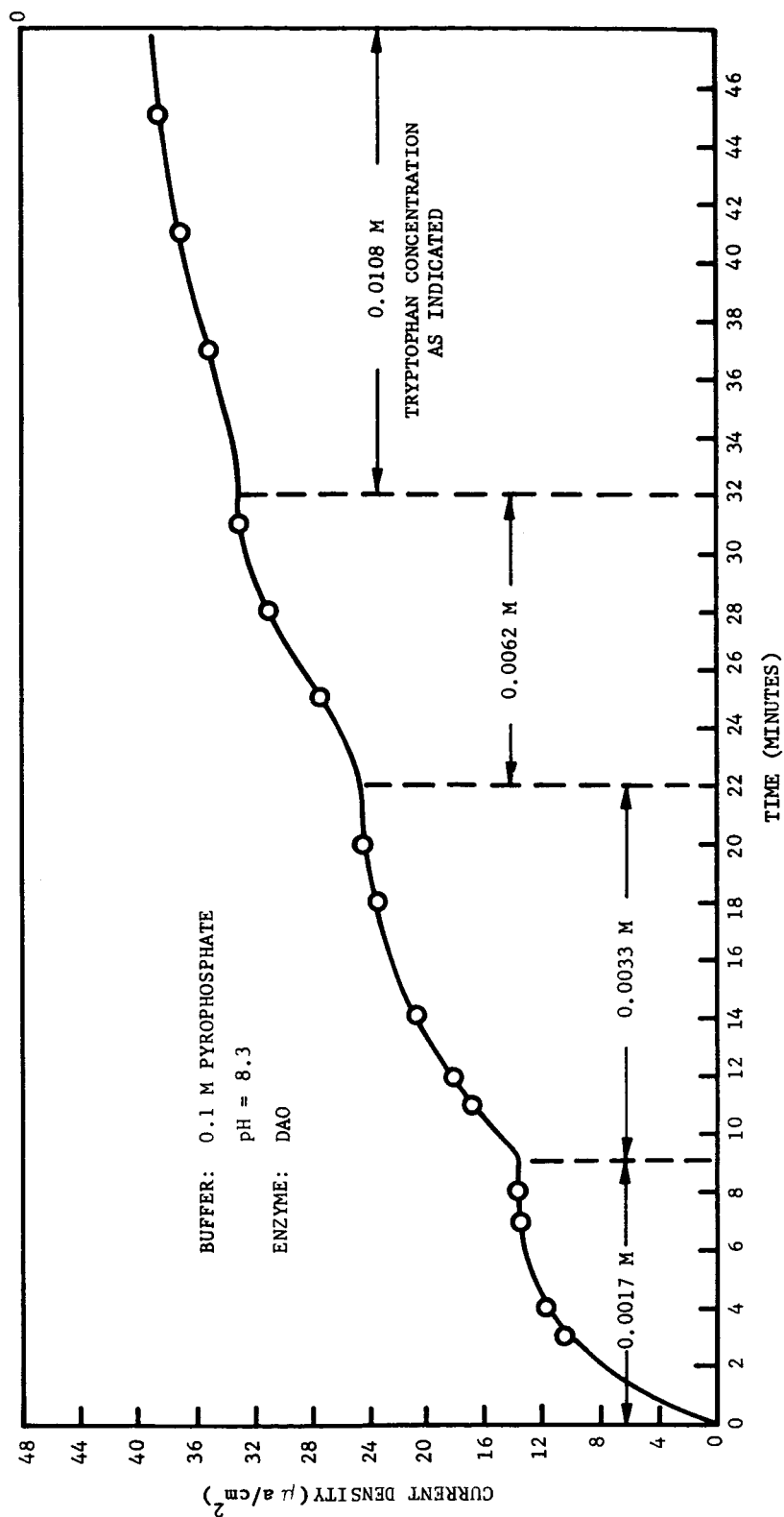
FIGURE 2. DEPENDENCE OF CURRENT UPON TRYPTOPHAN CONCENTRATION AT
CONSTANT ENZYME CONCENTRATION

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of about 10 minutes is obtained, after which a steady current is obtained approximately proportional to the square root of the tryptophan concentration.

The limiting value of the current is found to be significantly dependent upon enzyme concentration. This is shown by Figure 3 in which the reciprocal of enzyme concentration is plotted against the reciprocal of the current. This type of plot is according to standard procedure in enzyme chemistry except that reciprocal of the reaction rate is normally plotted instead of reciprocal of cell current. Data is shown for two identical runs, in each of which the enzyme concentration is incrementally increased by means of successive additions. Between additions, sufficient time was allowed for the current to achieve steady state value.

The straight line indicates that the dependence of rate on enzyme concentration is first order, as would be expected under conditions where the concentration of enzyme-substrate complex is small relative to the total substrate concentration. The displacement between the two curves is attributed to uncontrolled differences in the experimental conditions, such as perhaps in the degree of saturation of the anolyte with air. Complete interpretation of these observations and those of Figure 2 is not possible at this time, since a detailed kinetic study was not thought to be warranted.

One of the most striking observations made on this system is the large increase in current obtained aerobically over that obtained anaerobically, and this has proven to be a very important factor in identification of the pertinent electrode reaction. If a significant fraction of the current developed by this system arises from electrochemical oxidation of the reduced enzyme, admission of oxygen into the electrode chamber would be expected to compete with the electrode for the reduced enzyme, thus reducing the current. Such competition by oxygen would not be expected if the enzyme substrate complex, or some subsequent reaction product were the electroactive species. Figure 4
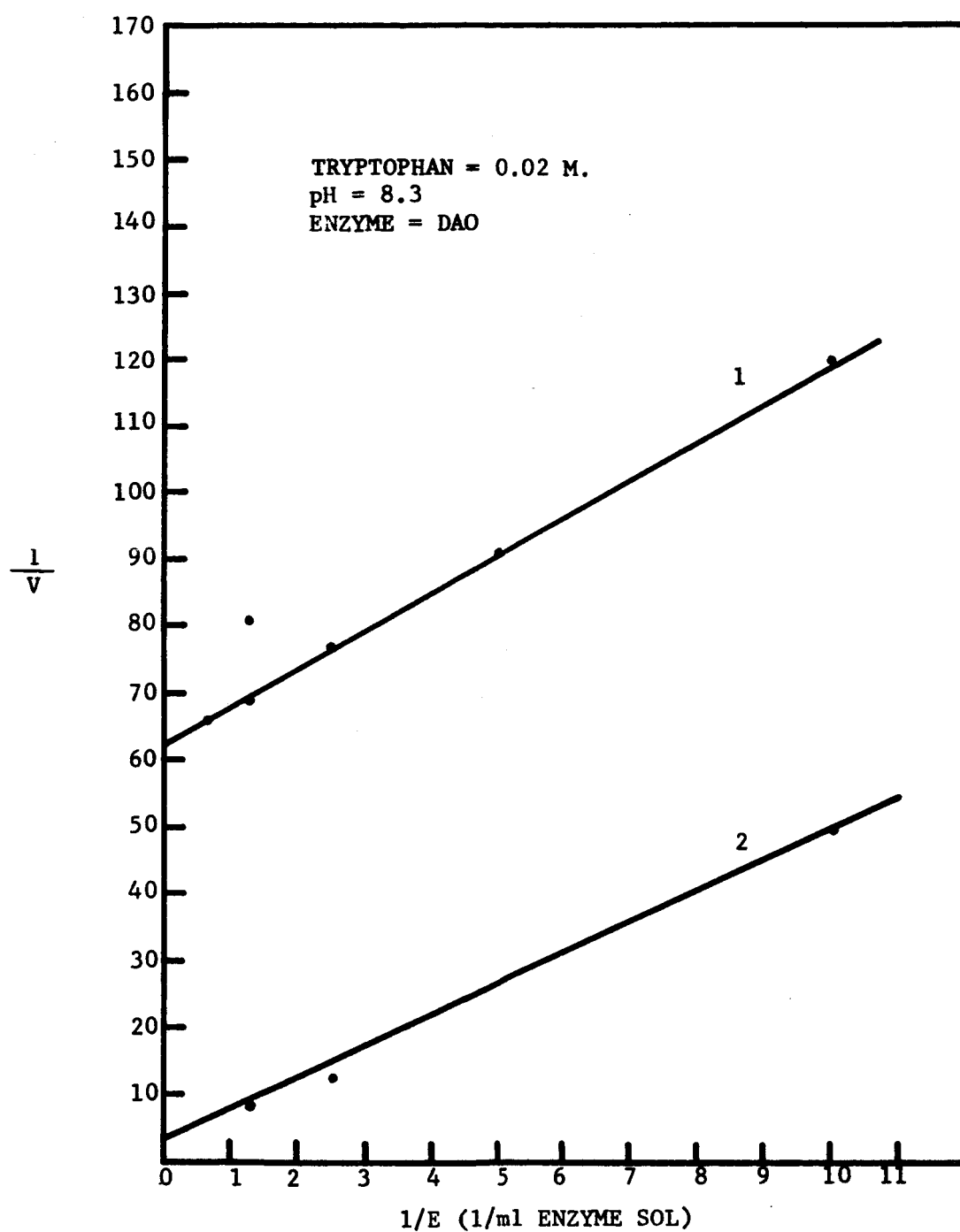
FIGURE 3. RECIPROCAL PLOTS OF ENZYME CONCENTRATIONS AND
CURRENTS AT CONSTANT TRYPTOPHAN CONCENTRATION

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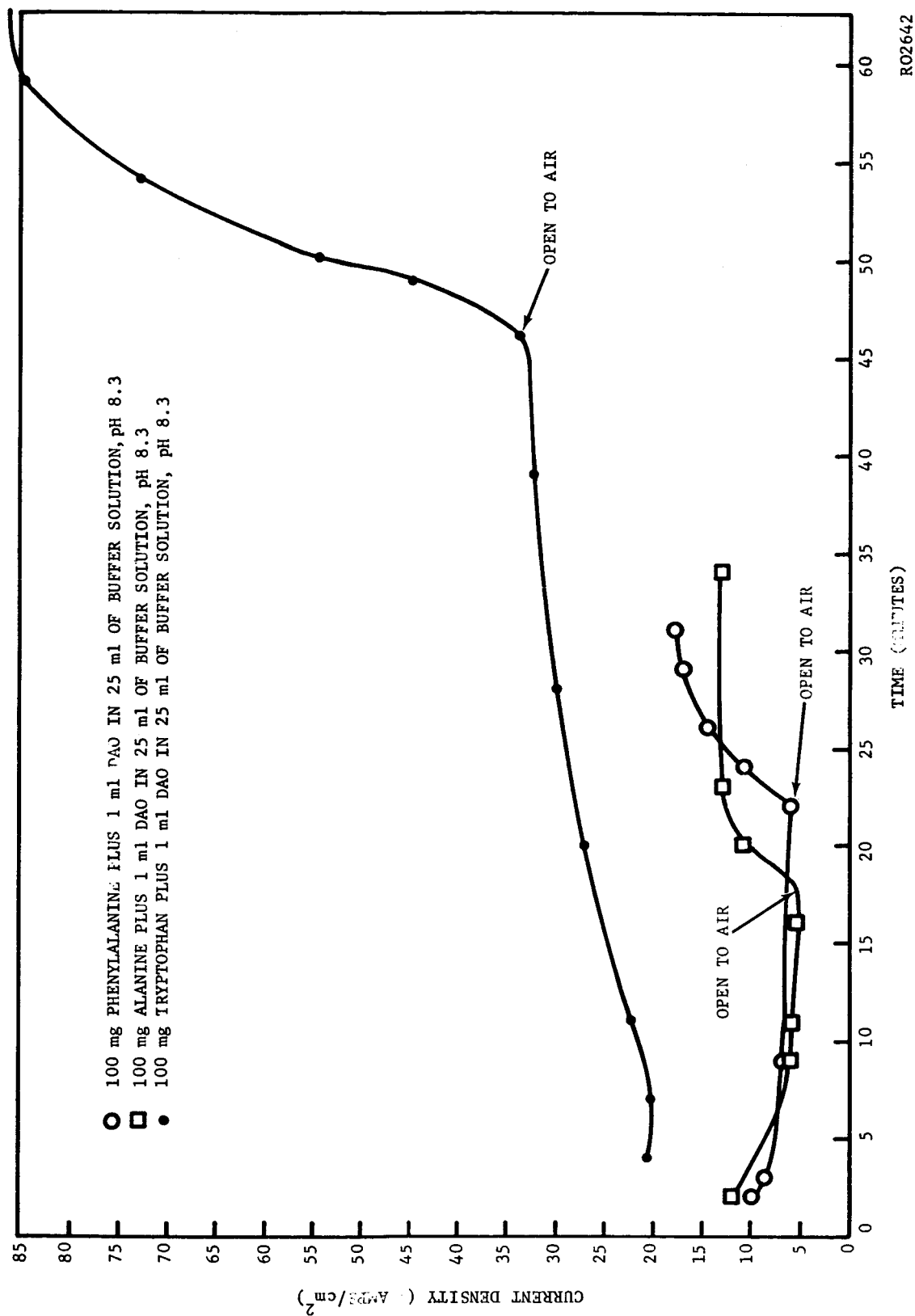
FIGURE 4. ELECTROCHEMICAL ACTIVITY OF DAO WITH DIFFERENT SUBSTRATES

O   100 mg PHENYLALANINE PLUS 1 ml DAO IN 25 ml OF BUFFER SOLUTION, pH 8.3
□   100 mg ALANINE PLUS 1 ml DAO IN 25 ml OF BUFFER SOLUTION, pH 8.3
●   100 mg TRYPTOPHAN PLUS 1 ml DAO IN 25 ml OF BUFFER SOLUTION, pH 8.3

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shows the effect upon cell current of admission of air to the electrode
compartment for three different substrates, namely phenylalanine, alanine
and tryptophane. It is seen that an increase in current occurs in all
cases, the largest increase being obtained with tryphophane. It must be
noted that this behavior does not preclude the participation of the
enzyme in the electrode reaction. However, in view of the above consid-
erations it is believed to strongly imply that a major part of the current
derives from electrochemical activity on the part of some
product of the aerobic enzymatic reaction.

The visible effect of air demonstrated here, in conjunction
with the chronopotentiometric studies (see Section 4.3) indicated the
possibility that the major part of current from the anaerobic tryptophan-
enzyme system might be due to accidental introduction of very small
amounts of oxygen into the solution. Measurements of electrical output,
made with special precautions to exclude oxygen during experimental
manipulation showed that available current in the DAO-tryptophan system
could be reduced to 1.1 to 2.0 $\mu a/cm^2$, in contrast to the 4 to 10 $\mu a/cm^2$
normally found. Results for the tyrosine-DAO system were found to be
similar.

With phenylalanine and alanine as substrates, admission of air
into the anode cell caused a much smaller increase in current output than
with tryptophan. This dependence upon substrate is consistent with con-
clusions to be described now as to the chemical species responsible for
the electrochemical reaction.

The reaction of DAO with an amino acid gives rise, initially,
to three important end products: a pyruvic acid derivative, NH_3 and H_2O_2.
If catalase is not present to destroy the peroxide as formed it can react
further with the pyruvate derivative resulting in decarboxylation and
formation of a shorter chain acid. Thus, three, and possibly four,
products could be responsible for the observed currents.

NH$_3$ was found to be completely inactive under the conditions used in these experiments when it was added in the form of ammonium chloride. In a separate experiment hydrogen peroxide, at 0.0048 M, the maximal concentration which might be found upon complete oxidation of the tryptophan, gave rise to substantial currents (see polarization curve of Figure 5). However, addition of catalase to the hydrogen peroxide destroyed the current in this experiment.

Addition of catalase to the DAO-alanine system also prevented any increase in current upon introducing air into the cell indicating that the entire alanine aerobic current was probably due to hydrogen peroxide formation. Addition of catalase to the tryptophan system did not significantly reduce the aerobic output and, in fact, in corresponding experiments under anaerobic conditions seemed to assist in maintaining the current. Thus, neither hydrogen peroxide nor ammonia would appear to be contributing significantly to the aerobic current in the DAO-tryptophan system. It follows that the electroactive species, in the case of tryptophan, is the reaction product indole pyruvic acid, and that if the corresponding product for other substrates is not electrochemically active, no effect upon current other than that attributable to peroxide is to be expected.

In order to examine this point further, studies were carried out on the activity in the electrochemical cell of the expected reaction products of three amino acid with DAO, namely tryptophane, tyrosine and phenylalanine. These product materials, indole pyruvic acid, p-hydroxyphenyl pyruvic acid, and phenyl pyruvic acid, were obtained from an outside source.

A stock solution was made by dissolving commercial indolepyruvic acid (IPA) in 0.1 M pyrophosphate buffer, (pH 8.3). This stock solution was added in increments to 25 ml of buffer and 0.6 ml of enzyme solution (1.0 mg/ml) in the electrochemical cell. Current readings at 0.200 v polarization potential were then made for each concentration. Since IPA
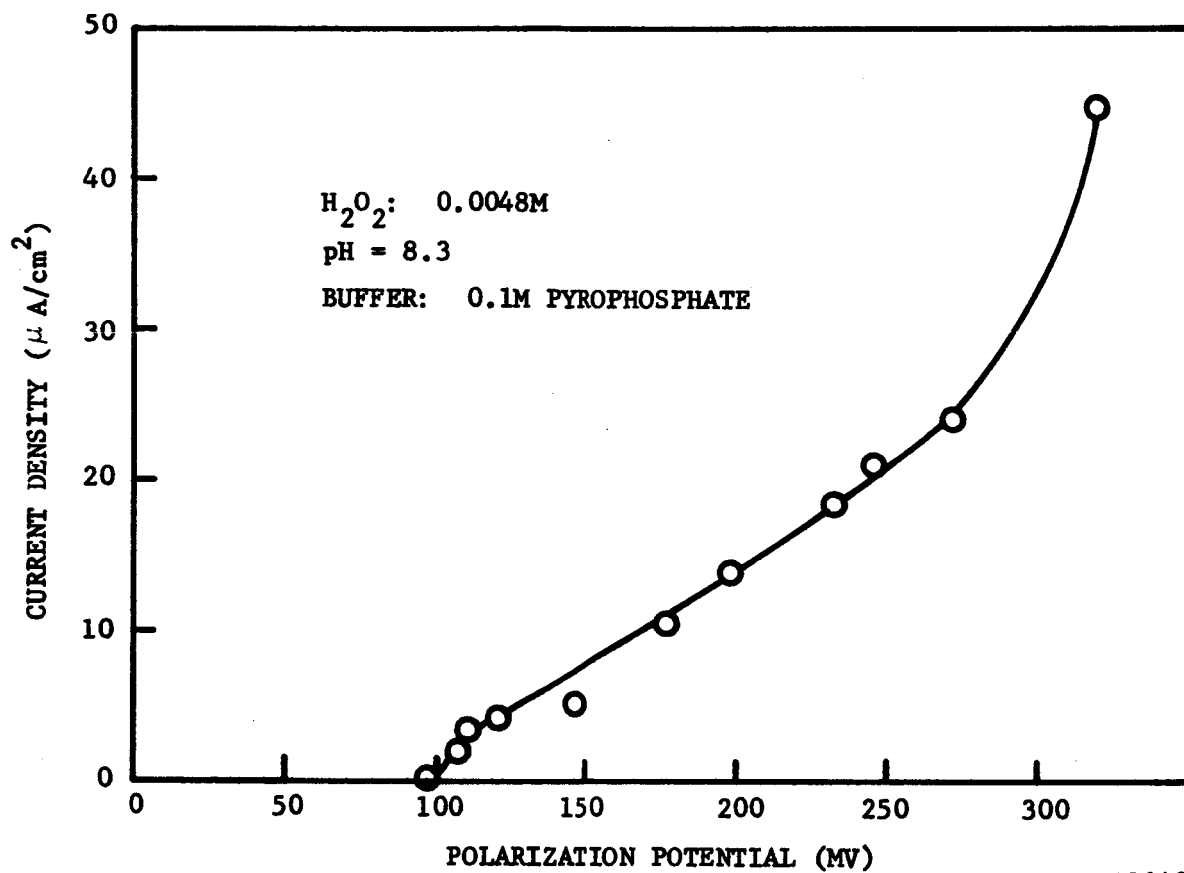
FIGURE 5. ELECTROCHEMICAL OXIDATION OF H_2O_2

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has only a limited solubility in the buffer used, concentrations above 0.0006 M were obtained by adding weighed quantities of the solid IPA to the cell solution and reading the current after complete solution of the sample.

Current was found to be linearly proportional to IPA concentration when obtained by addition of the stock IPA solution to the cell (Figure 6) but the two points at higher concentration, obtained by addition of solid IPA do not follow the same curve. The reason for this discrepancy will be discussed later. (See Section 4.5) No differences were found for the electrochemical oxidation of IPA carried out either aerobically or anaerobically.

The p-hydroxphenyl pyruvic acid and phenyl pyruvic acid were tested similarly and the results are given in Table 1. Currents obtained with phenyl pyruvate were negligible, in line with the low currents found for phenylalanine oxidation (Figure 4). Despite low concentrations of hydroxyphenyl pyruvate, significant currents, proportional to concentration, were obtained. Tyrosine was also capable of supporting significant currents during reaction with DAO in the electrochemical cell (Figure 8, previous quarterly report).

4.2 ANALYTICAL STUDIES ON THE D-AMINO ACID OXIDASE SYSTEM AND CORRELATION WITH ELECTRICAL OUTPUT

In order to establish the extent of oxidation of the amino acids in the electrochemical cell, to determine whether levels of indole pyruvate derived from tryptophan could account for total cell current, and to establish electrochemical yields and efficiencies, it was desirable to study the relative levels of substrate and product in the electrochemical cell under load conditions. Preliminary spectrophotometric observations revealed the progressive formation of a new shoulder or absorption peak in the spectra of the aromatic amino acids during oxidation by DAO in dilute solution. For tryptophan (Figure 7) this peak fell at 306 mμ, for tyrosine at 295 mμ and for phenylalanine at 285 mμ. It appeared feasible
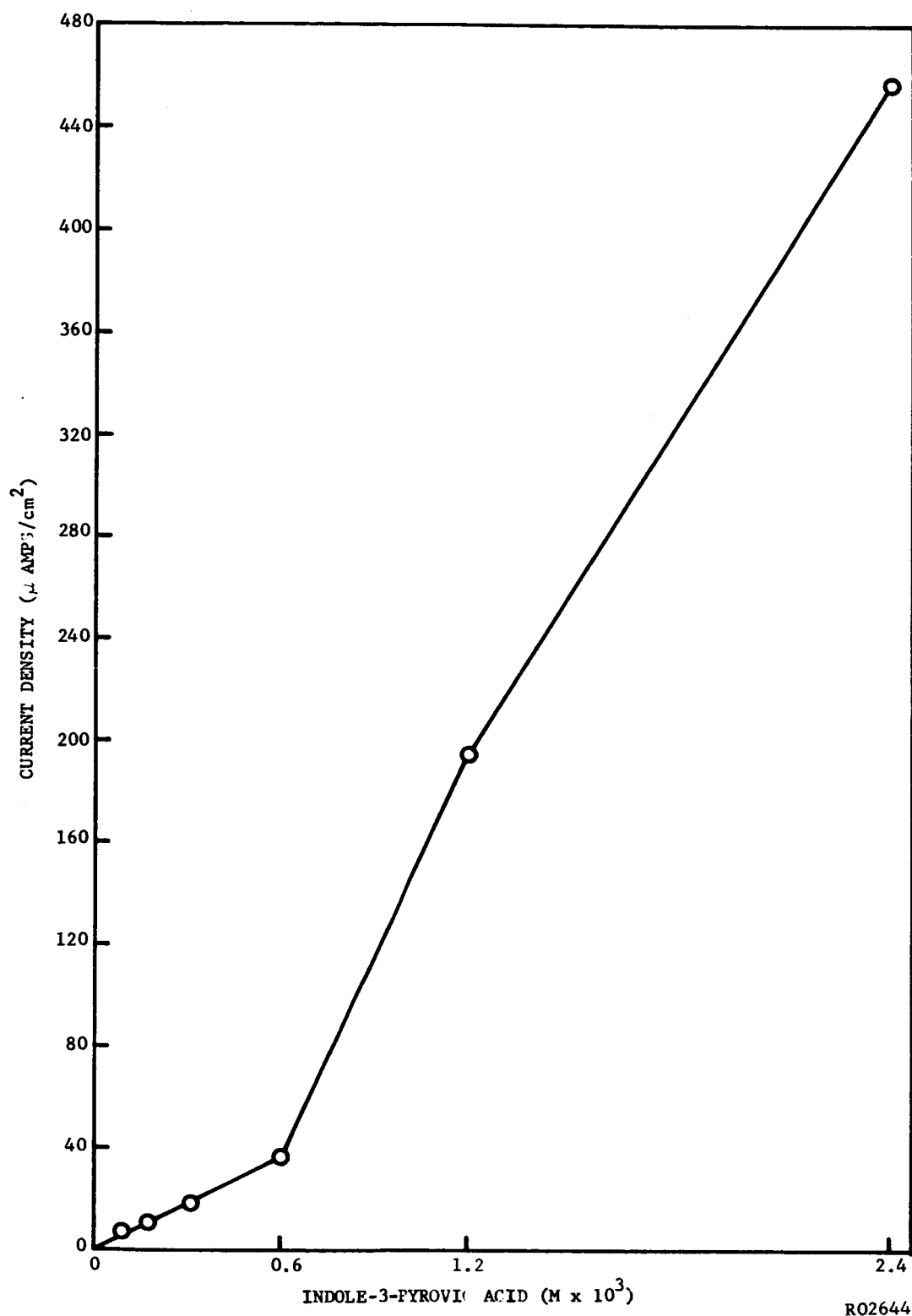
FIGURE 6.   CURRENT FROM INDOLE-3 PYRUVIC ACID

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TABLE 1

ELECTROCHEMICAL OXIDATION OF AROMATIC PYRUVIC ACIDS

Phenylpyruvic Acid (PPA)

Conditions: 0.1 M pyrophosphate buffer, pH 8.3,
anolyte volume-25 ml, Nitrogen gas phase

Cell Contents	$V_o(mv)^*$	$V_c(mv)^*$	$I_c(\mu a/cm^2)^*$
Buffer	+209	unstable	negligible
14.4 μg/ml PPA	+210	unstable	negligible
28.8 μg/ml PPA	+215	unstable	negligible
57.6 μg/ml PPA	+215	unstable	negligible
115 μg/ml PPA	+213	unstable	negligible

p-Hydroxyphenyl Pyruvic Acid (HPA)

Conditions: as above

Cell Contents	$V_o(mv)$	$V_c(mv)$	$I_c(\mu a/cm^2)$
Buffer	+234		
18 μg/ml HPA	+150		
35 μg/ml HPA		+200	3.5
65 μg/ml HPA		+200	5.5
114 μg/ml HPA		+200	7.9

* V_o - open circuit potential

V_c - closed circuit potential

I_c - load current

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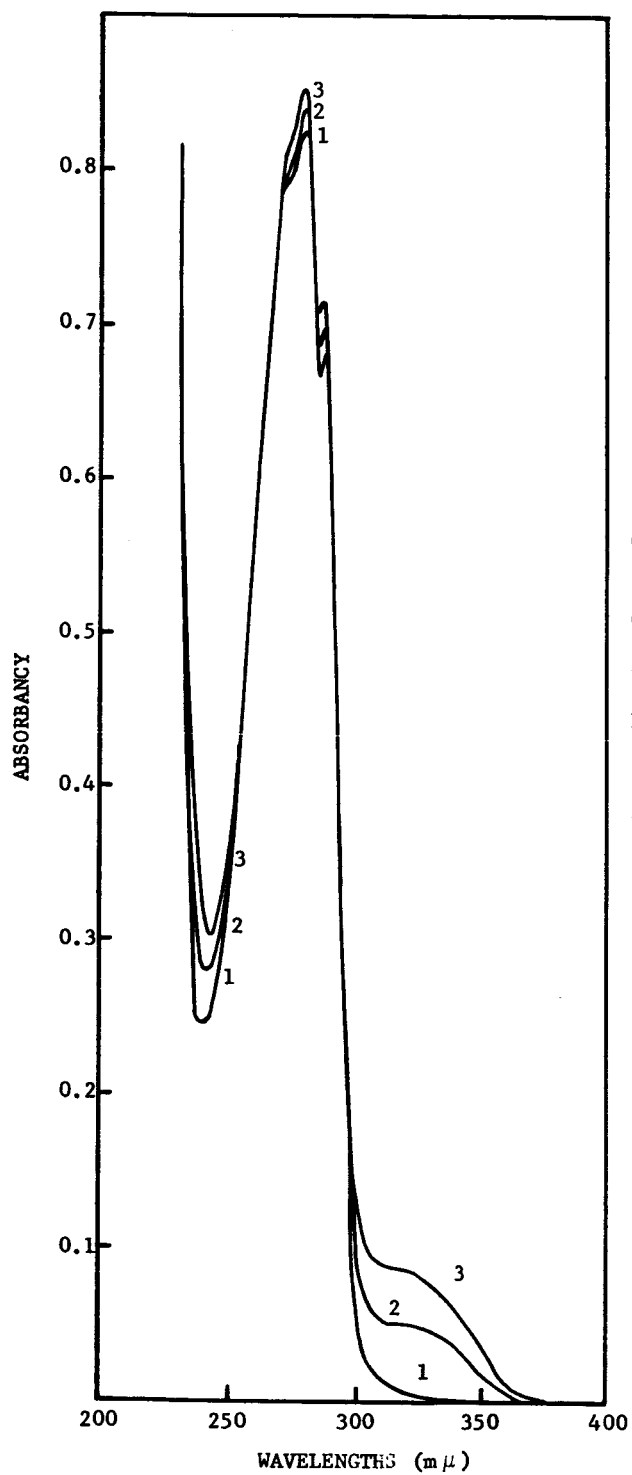
CURVE 1.   IMMEDIATELY AFTER
           ENZYME ADDITION

CURVE 2.   20 MINUTES AFTER

CURVE 3.   40 MINUTES AFTER

BUFFER: PYROPHOSPHATE

pH   =   8.3

TRYPTOPHAN CONCENTRATION 0.005M

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to develop a method for direct analysis of the oxidation products using these peaks. The method is as described below for study of the DAO-tryptophane system.

A standard absorption curve for the determination of IPA concentration in the electrochemical cell was. prepared as follows. Both reference and standard absorption cells were prepared containing 0.005 M D-tryptophane. A solution of 0.005 M indole pyruvic acid was also prepared. The latter solution was used to replace aliquots removed from the measured volume of tryptophane solution in the sample cell so as to obtain known concentrations of IPA in the presence of tryptophan. Spectral scans were made from 350 mμ to 230 mμ for each value of IPA using a 1 mm light path, obtained through the use of optical spacers in a 10 mm cell. The concentration of 0.005 M tryptophan was chosen since this approached the maximum concentration permissible with a 1 mm optical path before becoming opaque in the region of the tryptophan peak at 279 mμ. This concentration was about one-fourth that previously used in the electrochemical cell. In all cases the solution contained pyrophosphate buffer to provide a pH of 8.3.

A typical spectrum for this system is given in Figure 8 while the calibration curves at several wavelengths are given in Figure 9. The curves for the difference maxima observed at 305 mμ and 234 mμ show good linearity over the region investigated. The curve obtained at 265 mμ would probably be unsuitable for analytical purposes.

Figure 10 shows the use of this analytical method in a study of the rate of amino acid oxidation in a stoppered Cary cuvette at pH 8.3 by DAO. A linear curve for product formation was found during a period of 20 minutes. On the other hand, tyrosine and phenylalanine did not conform to simple kinetics under these circumstances.

An investigation of the accuracy attainable with this analytical method was carried out by determining the indole pyruvic acid content of a known solution and comparing the "found" and "added" amounts. Tryptophan
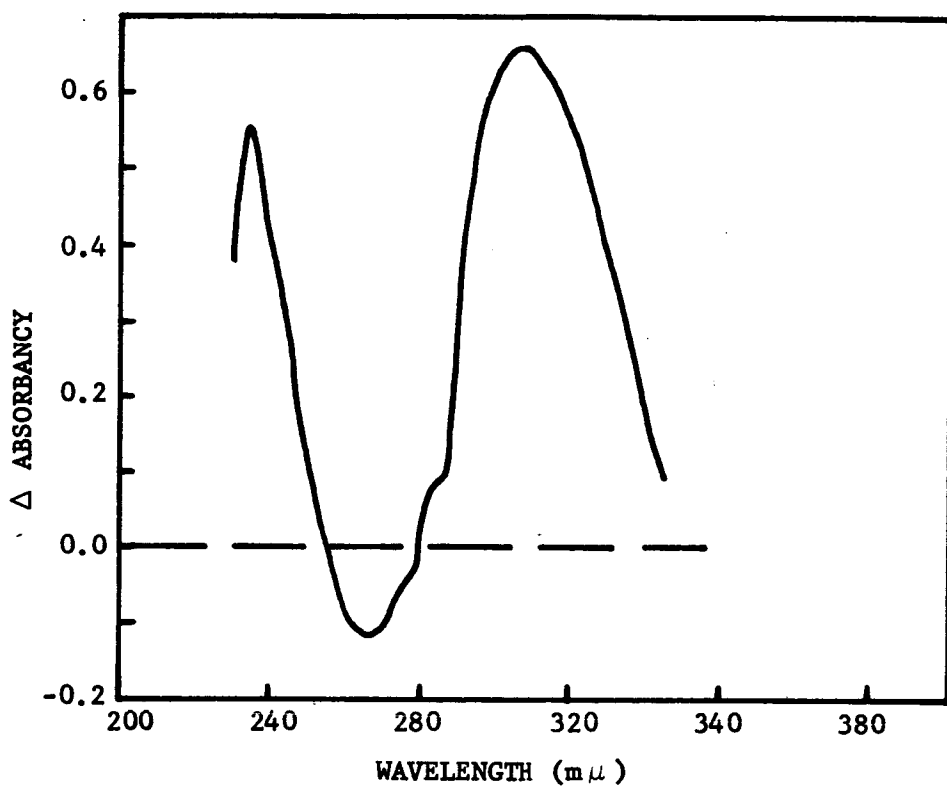
REFERENCE: 0.005M
TRYPTOPHAN

SAMPLE: 0.0025M IPA IN
0.0025M
TRYPTOPHAN

FIGURE 8. DIFFERENCE SPECTRUM FOR IPA - TRYPTOPHAN
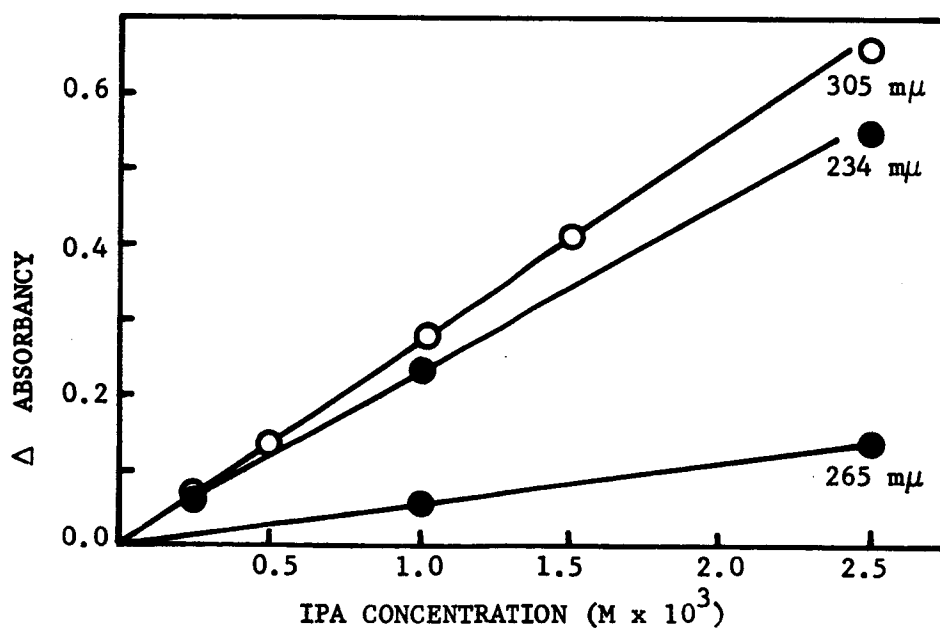
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FIGURE 9. CALIBRATION CURVES FOR IPA CONCENTRATION
IN PRESENCE OF TRYPTOPHAN

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	A. TRYPTOPHAN		B. TYROSINE		C. PHENYLALANINE	
	SAMPLE CELL	REF CELL	SAMPLE CELL	REF CELL	SAMPLE CELL	REF CELL
AMINO ACID*	2.0 ml	2.0 ml	3.0 ml	3.0 ml	2.0 ml	2.0 ml
BUFFER	0.5	0.5	0	0	0.5	0.5
DAO (ca 0.05 mg/ml)	0.5	0	0.5	0	0.5	0
WATER	0	0.5	0	0.5	0	0.5

*TRYPTOPHAN AND TYROSINE @ 0.002 M: PHENYLALANINE AT 0.02 M
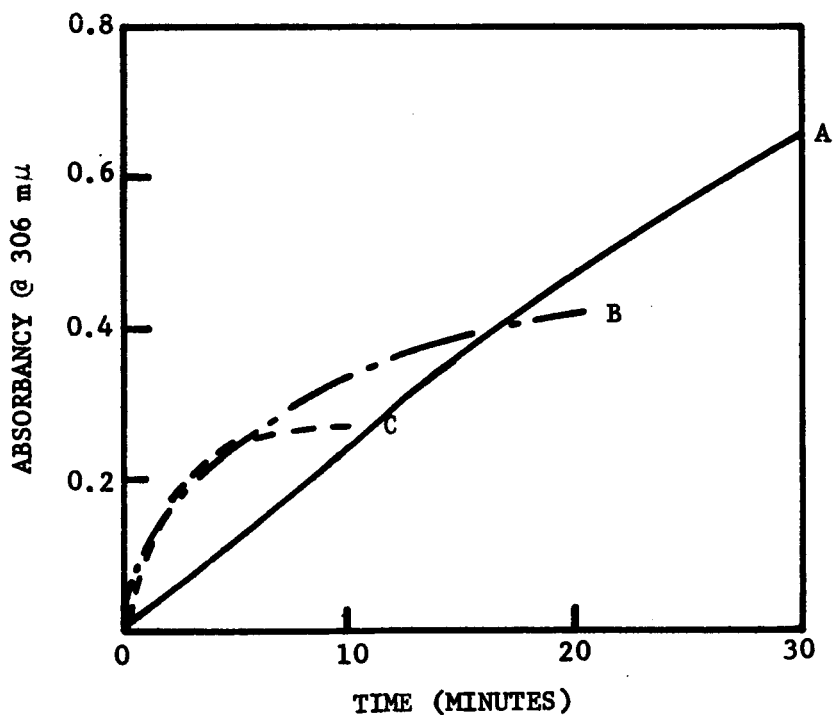
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FIGURE 10. OXIDATION OF AROMATIC AMINO ACIDS BY D-AMINO ACID OXIDASE

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solutions were introduced into the electrochemical cell with and without enzyme (0.005 M tryptophan, 1.0 ml of purified DAO). Aliquots of the cell solution were then withdrawn and replaced with similar portions of 0.005 M IPA. A nitrogen atmosphere was maintained at all times over the solutions and the solutions were introduced and withdrawn with syringes previously flushed with nitrogen. Finally, 0.6 ml of the cell solution was transferred to a cuvette via syringe and scanned in the ultra-violet region, as above, using a 1 mm light path. The spacer which was used to give the short path also served the purpose of greatly reducing the area of the air-solution interface thus minimizing increase in IPA due to enzymatic oxidation of tryptophane during the short scanning period.

Differences found between added and found values are shown in Table 2. The determination at concentrations in the range of 0.0002 M and higher appears at this time to be reasonably reliable for the purposes intended.

An example of the application of this analytical procedure to an actual electrochemical run is illustrated in Figure 11. In this case, DAO and catalase (the latter being added to prevent accumulation of hydrogen peroxide and destruction of the IPA) are added to 0.005 M tryptophan at time zero. An aliquot was taken immediately for photometric analysis. Some time later, under anaerobic conditions, the circuit was closed to draw current at the regular electrode potential of 0.200 v. (vs SCE). At this time a second spectrophotometric sample was taken and sampling subsequently continued at intervals during the remainder of the run. Apparent IPA had reached about 0.0001 M at the beginning of the discharge. When the circuit was opened once during the anaerobic phase and then closed the current showed a short surge above the previous current although the apparent IPA concentration had not changed during the interim. Upon opening the system to air, the current immediately rose and IPA also rose commensurately. Following introduction of nitrogen once more, the current remained constant although the IPA values increased slightly for a while,
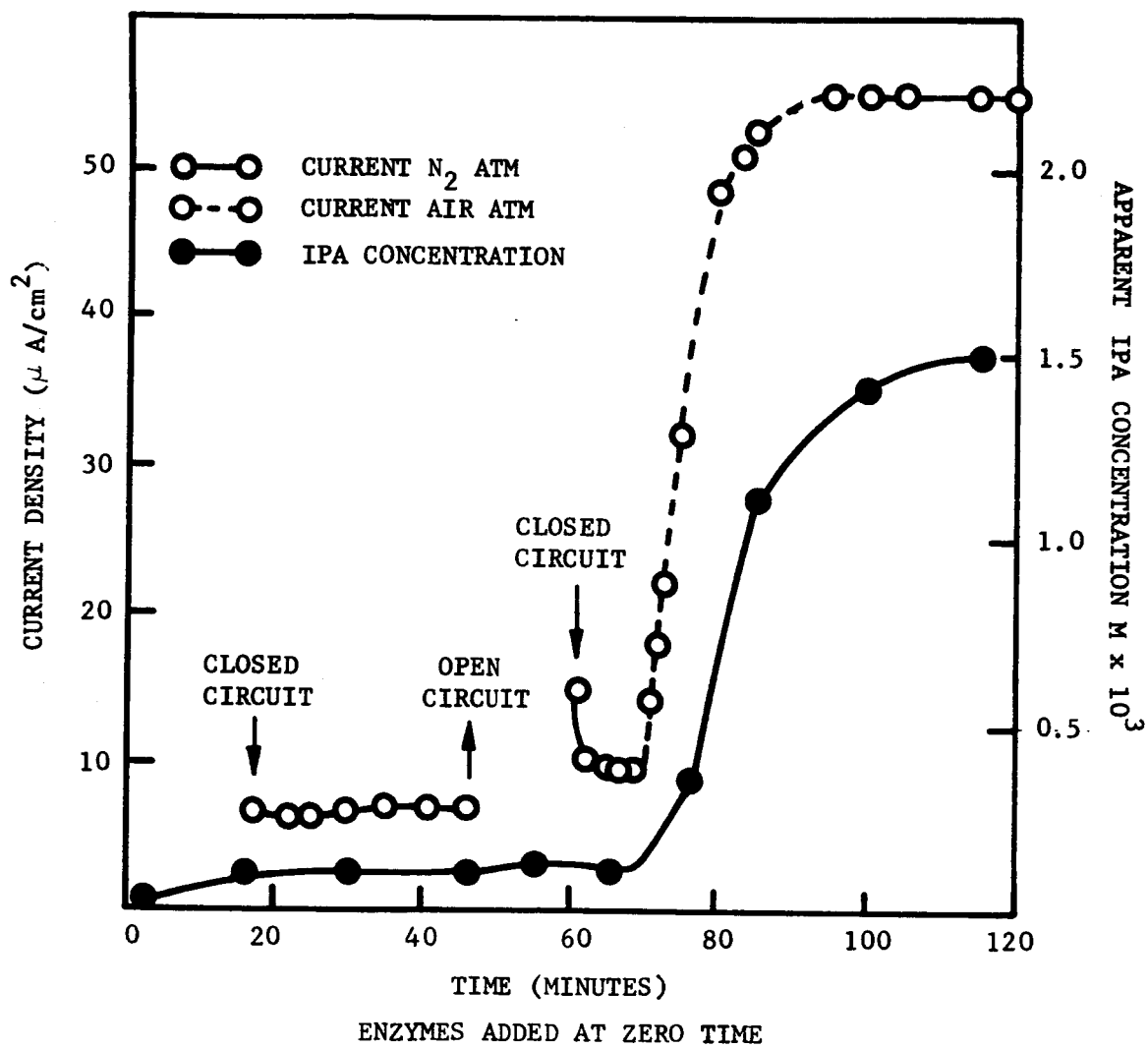
FIGURE 11.   RELATION OF ENZYMATICALLY FORMED IPA TO CURRENT

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presumably due to residual oxygen in the system. Almost direct linear
relation between IPA concentration and current appears to exist in the
lower regions of concentration under aerobic conditions. At higher values,
the current falls off in relation to total IPA. In the anaerobic phase,
where IPA is in very low concentration, the value found is probably con-
siderably higher than that actually in the cell since, in the manipula-
tions involved in transferring samples from cell to cuvette a short period
of contact with air occurs allowing for some rapid oxidation. This could
probably be avoided by maintaining a nitrogen atmosphere over the cuvette
during such manipulations.

TABLE 2

COMPARISON OF ADDED IPA AND FOUND IPA
DETERMINED BY SPECTROPHOTOMETRIC ANALYSIS

IPA Concentrations ($M \times 10^3$)

Calculated from added IPA	Spectrophotometrically determined		Current Densities ($\mu A/cm^2$)	
	+ Enzyme	Minus Enzyme	+ Enzyme	Minus Enzyme
0	0.02	0.01	3.7	-
0.25	0.25	0.20	13	10
0.84	0.76	0.70	30	21

4.3 ELECTROCHEMICAL ACTIVITY OF THE TRYPTOPHANE-DAO SYSTEM

Anodic chronopotentiograms of $5 \times 10^{-3} M$ tryptophan in 0.1M sodium
pyrophosphate ($Na_4P_2O_7$) buffer solution of pH 8.3 were obtained for
various currents ranging from 20 to 100 μa. Typical curves illustrated
in Figure 12 show that the potential halts are essentially independent
of whether or not the solution is stirred during the recording of the
chronopotentiograms. Anson and Schultz (4) reported that the transition
time for the oxidation of oxalic acid at the platinum electrode was
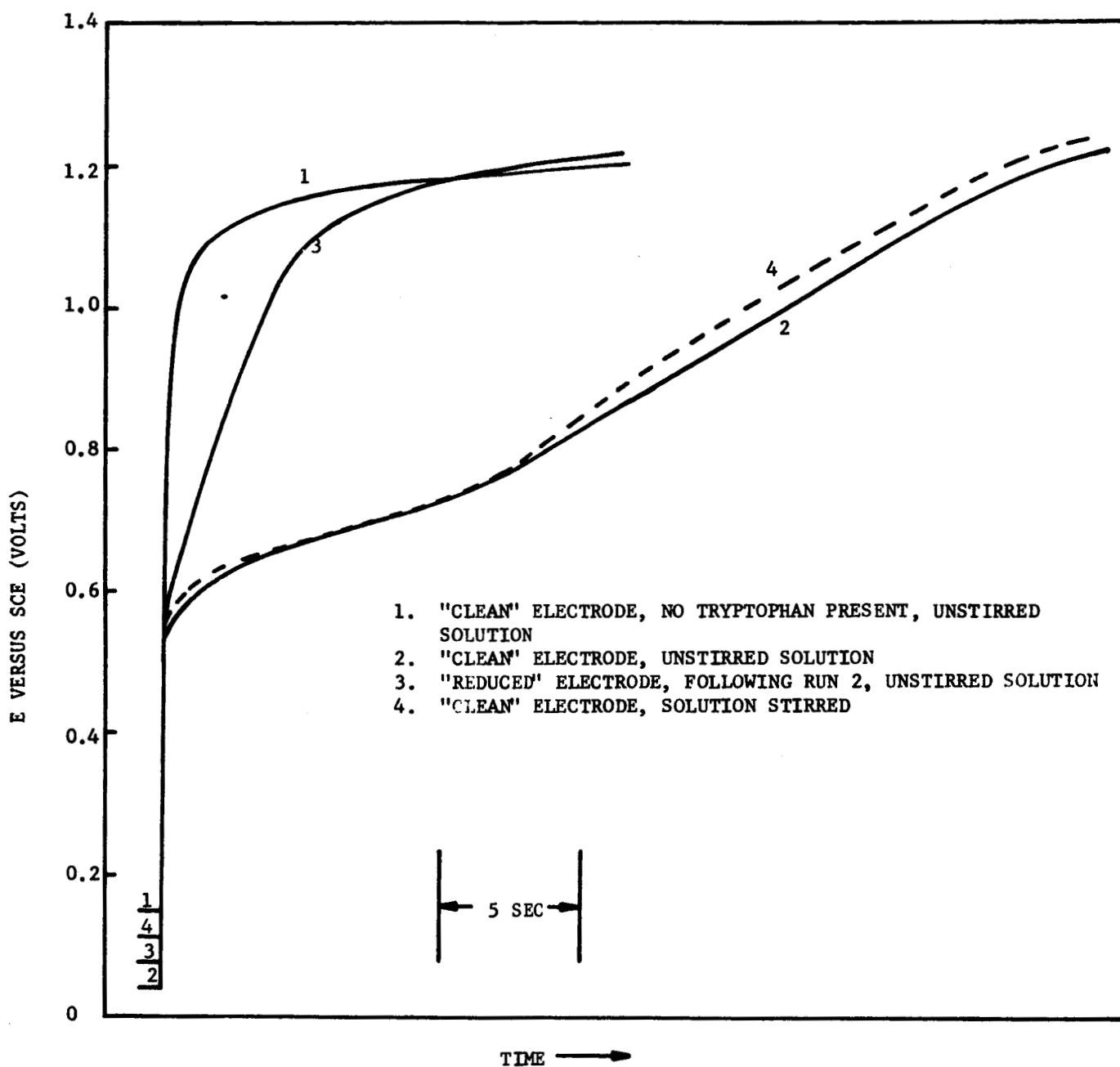independent of stirring in buffered solution above pH 3. They were able

FIGURE 12. ANODIC CHRONOPOTENTIOGRAM FOR OXIDATION OF 5×10^{-3} M D-TRYPTOPHAN IN 0.1 M $Na_4P_2O_7$ BUFFER SOLUTION, pH 8.3, i = 100 μa

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to account for this behavior on the basis of an oxalic acid adsorption mechanism and on the fact that extensive oxidation of platinum occurs at pH values above 3. It is plausible that an adsorption mechanism is also involved in the oxidation of tryptophan.

Further support of the adsorption mechanism in the oxidation of tryptophan was derived from the plot of $i\tau^{\frac{1}{2}}$ vs i (Figure 13). According to the diagnostic criterion developed by Reinmuth (5) a positive slope in this plot predicts that the reaction scheme involves an adsorption process.

The absence of a potential arrest in Curve 3 (Figure 12) reveals that no appreciable oxidation of tryptophan occurs on the "reduced" electrode. It is apparent from this observation that adsorbed oxidation products from the previous experimental run inhibit the oxidation of tryptophan in successive runs. Similar behavior was observed on a "reduced" electrode with tryptophan in sulfuric acid (pH=0).

The oxidation behavior of 5×10^{-3}M indole-3-pyruvic acid (IPA) in 0.1M $Na_4P_2O_7$ buffer solution was examined chronopotentiometrically, using a "clean" electrode. The oxidation wave was found to occur at $E_{\tau/4}$ = 0.61 to 0.63 volts vs SCE. Transition times were determined for a range of currents (25 to 60 μa) in an unstirred solution. The $i\tau^{\frac{1}{2}}$ values are tabulated in Table 3. The constancy of $i\tau^{\frac{1}{2}}$ values (within 5%) indicates that the oxidation process is diffusionally controlled. Since the $i\tau^{\frac{1}{2}}$ value was found to be a constant for the oxidation of IPA, it was of interest to determine whether the electrochemical process was reversible or irreversible. Distinction (6) between the two processes can be made by plotting $\log \frac{\tau^{\frac{1}{2}}-t^{\frac{1}{2}}}{t^{\frac{1}{2}}}$ vs E and $\log \left[1-\frac{t}{(\tau)}^{\frac{1}{2}}\right]$ vs E. If the first graph yields a straight line, then the process is reversible. For an irreversible process the second graph will give a straight line. Potential-time curves obtained with IPA were treated according to the two methods but failed to show a straight line relationship in either case. Thus, it is not possible to conclude as to the nature of the electrode process.
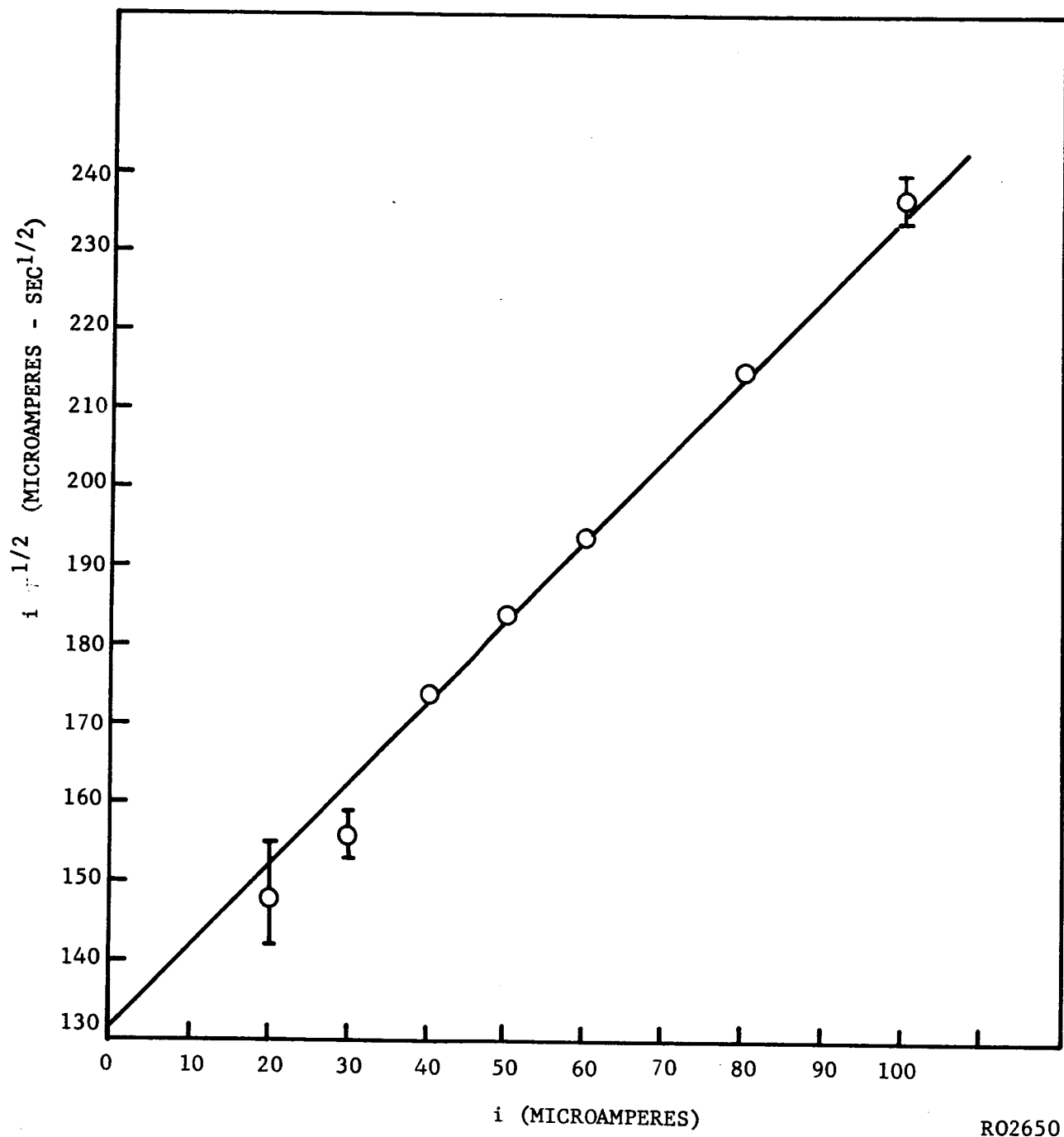
FIGURE 13. VARIATION OF $i\tau^{1/2}$ VERSUS i FOR 5 x 10^{-3} M TRYPTOPHAN IN UNSTIRRED 0.1 M $Na_2P_2O_7$ BUFFER SOLUTION

TABLE 3

CHRONOPOTENTIOMETRY OF INDOLE-3-PYRUVIC ACID

$i,\mu a$	T,sec.	$iT^{\frac{1}{2}},\mu a\text{-sec.}^{-\frac{1}{2}}$
25	167	324
30	134	348
35	89	328
40	74.4	346
50	51.6	360
60	28.8	322

Ave: 338 $\left(\mu a\text{-sec.}^{-\frac{1}{2}}\right)$
Std. deviation: 15.5

It is also of interest to determine whether or not poisoning of the electrode results from oxidation of IPA, as was reported previously to be the case for tryptophane. This is shown by the chronopotentiograms of Figure 14. Two cases are shown in each of which a run carried out on a "clean" electrode is followed by a second run, the electrode being merely "reduced" in between. Disappearance of the potential halt shows that poisoning occurs as a consequence of the first oxidation run in both cases. It was found here, and also with tryptophane, that poisoning does not occur if potentials in the initial run with the "clean" electrode do not at any time exceed values of about 0.9 volts vs SCE.

In a stirred solution of IPA, an oxidation wave was observed at 0.12v for a current-value of 50 μa. In the sixth monthly progress report it was suggested that this oxidation wave was due to impurities present in the acid. However, as will be discussed in a following section, there is reason to believe that when dissolved in buffer solution, IPA consists of an equilibrium mixture of two tautomeric forms corresponding probably to the enol and keto structures. It was established, moreover, that the absorbancy ascribed to the enol structure decreased progressively with time. It is conceivable that the potential halt found at 0.12v may be due to the preferential oxidation of one of the tautomeric forms. To examine this possibility, chronopotentiometric measurements were taken on
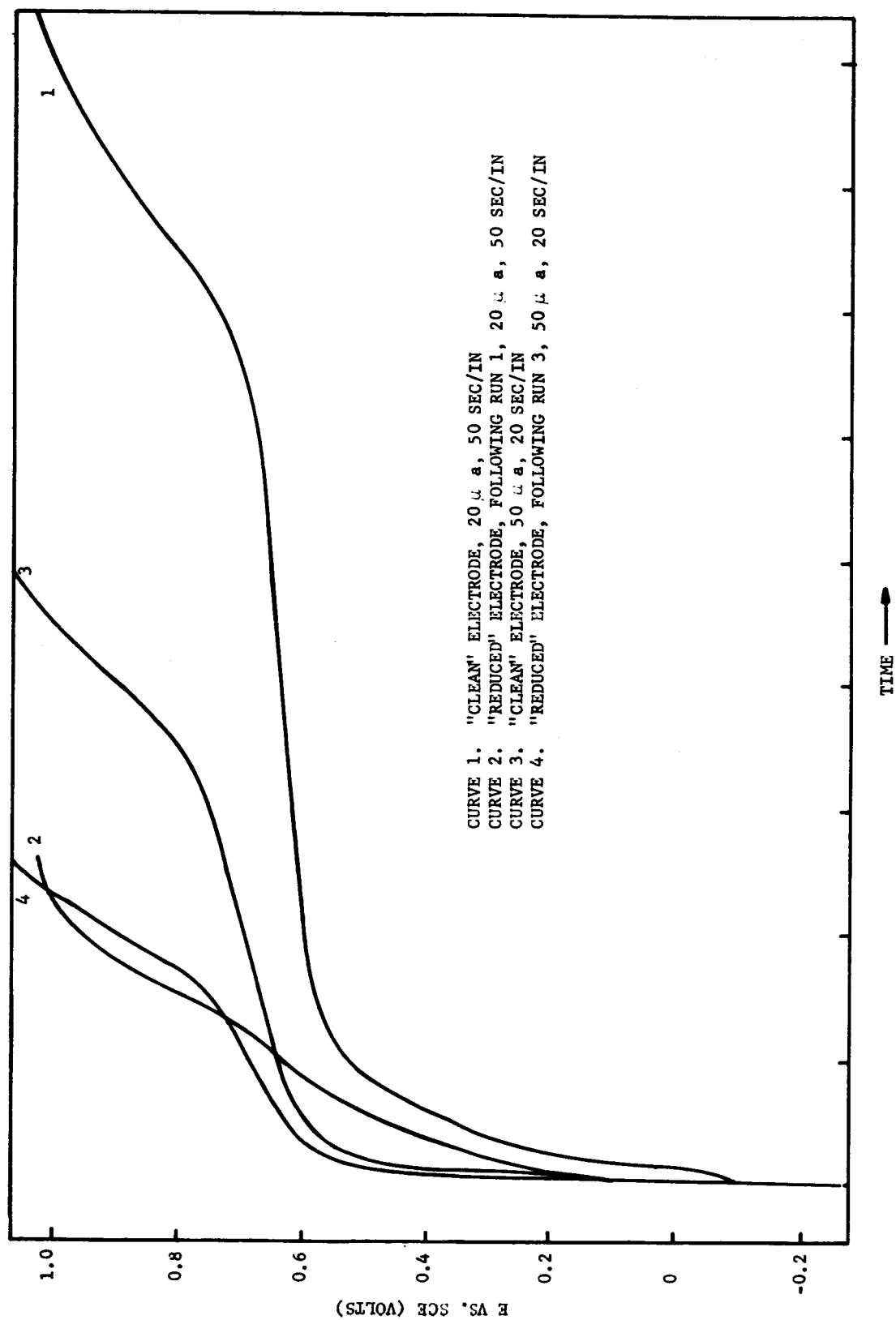
FIGURE 14. TYPICAL ANODIC CHRONOPOTENTIOGRAMS OF 5 x 10^{-3} M IPA - 0.1 M Na$_4$P$_2$O$_7$ SOLUTION ON "CLEAN" AND "REDUCED" ELECTRODE

CURVE 1. "CLEAN" ELECTRODE, 20 μ a, 50 SEC/IN
CURVE 2. "REDUCED" ELECTRODE, FOLLOWING RUN 1, 20 μ a, 50 SEC/IN
CURVE 3. "CLEAN" ELECTRODE, 50 μ a, 20 SEC/IN
CURVE 4. "REDUCED" ELECTRODE, FOLLOWING RUN 3, 50 μ a, 20 SEC/IN

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a freshly prepared solution. The solution was unstirred during the recording of the chronopotentiograms. Curves 1-3 in Figure 15 show the typical behavior of the potential arrest with time elapsed after initial dissolution of the acid. The fact that the potential arrest decreases with time strongly suggests that the first oxidation wave is due to the enol form and not to impurities in the acid.

The chronopotentiograms of D-tryptophan - D-amino acid oxidase system in buffer solution are shown in Figures 16 and 17. Displacement of the curves is imposed electronically and does not represent a difference in the respective potentiograms. The similarity in form of Curves 1-4 indicates that the reduced enzyme oxidation does not occur at the electrode. With the addition of an intermediate electron acceptor for the enzyme, in this case oxygen, the bio-electrochemical reaction is greatly enhanced as shown by Curves 5 and 6 in Figures 16 and 17. It appears from these studies that either the reduced enzyme does not become oxidized at the electrode, or if it does much higher enzyme concentrations than those used in the present investigation would be required to produce measurable current from such oxidation.

4.4 IDENTIFICATION OF ELECTROCHEMICAL OXIDATION PRODUCTS IN THE TRYPTOPHANE-DAO SYSTEM

Identification of products of the electrochemical reaction are complicated both by the small amount of material involved in the identification and the probability that both the reactant and product will be oxidizable by air. Consequently, in order to obtain anything resembling the quantification needed for energy and material balance studies, the identifications and measurements, up through the stage of separation of all the components, would have to be completely anaerobic.

Therefore, it appeared that the best possibility for obtaining rapid separation of the reaction substrates and products under controlled conditions is offered by an analytical method based on zone electrophoresis.
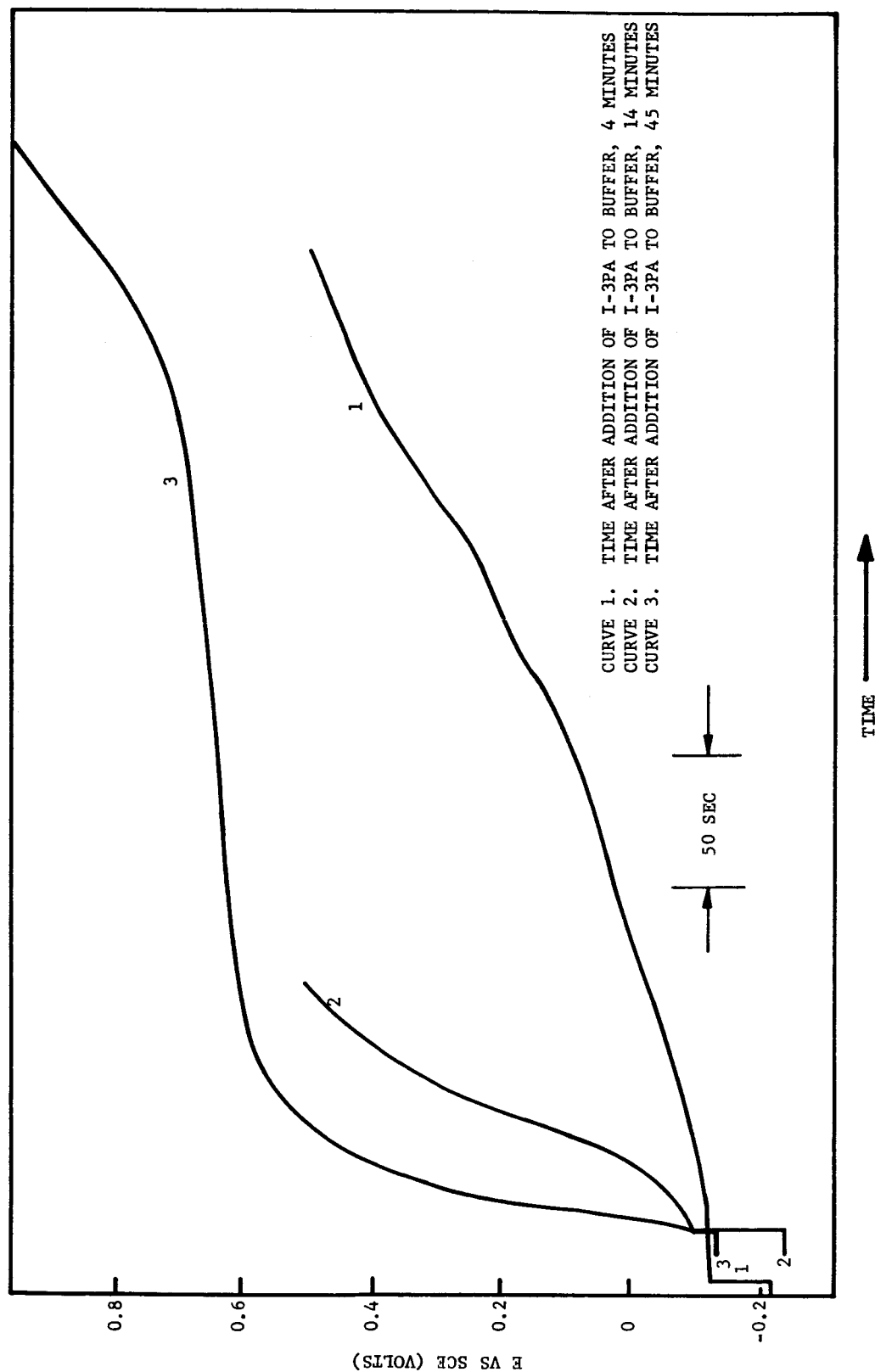
FIGURE 15. ANODIC CHRONOPOTENTIOGRAM OF 5×10^{-3} M INDOLE-3 PYRUVIC ACID IN 0.1 M $Na_4P_2O_7$ BUFFER SOLUTION OF pH 8.3, 20 μ a, SOLUTION UNSTIRRED
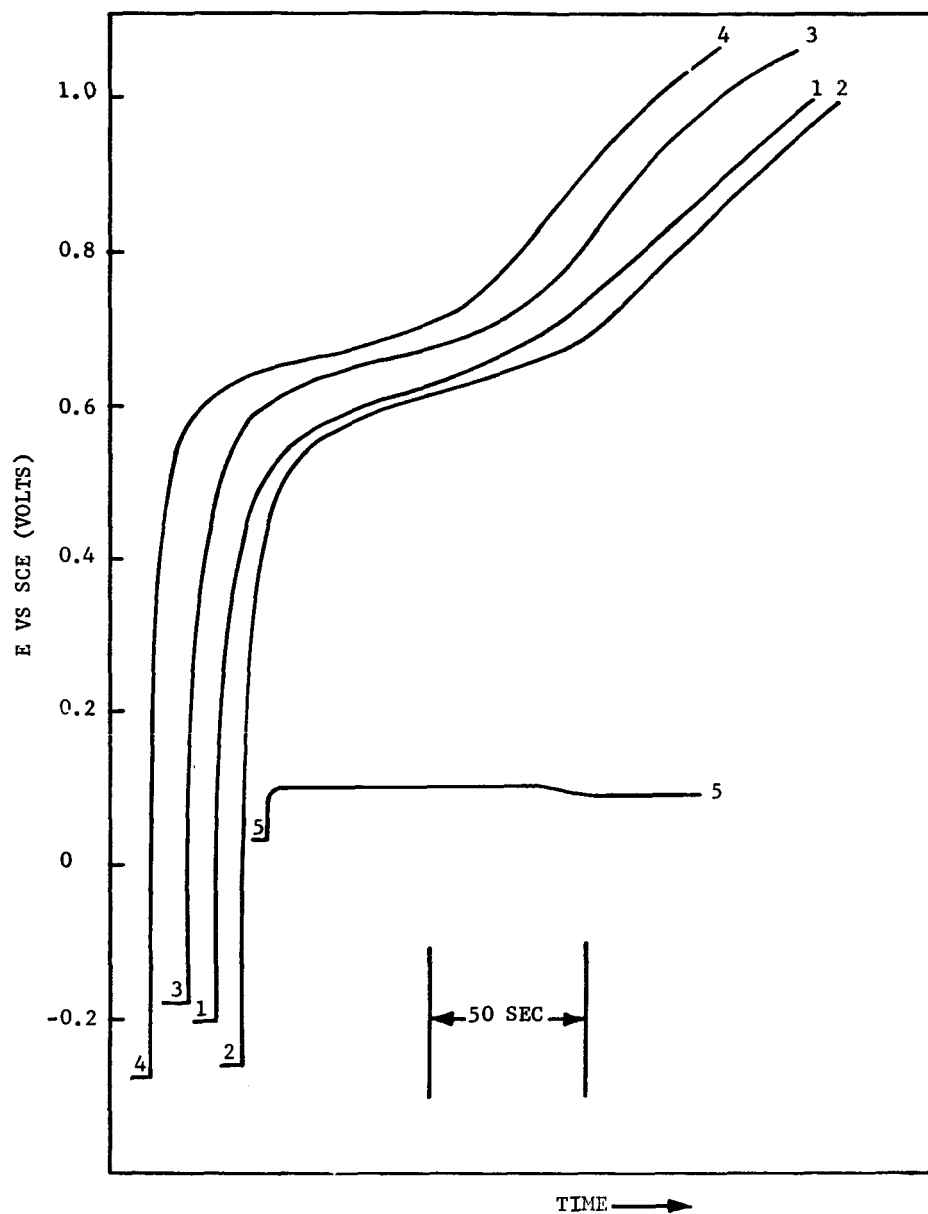
CURVE 1. TIME AFTER ADDITION OF I-3PA TO BUFFER, 4 MINUTES
CURVE 2. TIME AFTER ADDITION OF I-3PA TO BUFFER, 14 MINUTES
CURVE 3. TIME AFTER ADDITION OF I-3PA TO BUFFER, 45 MINUTES

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CURVE 1. 5 x 10^{-3}M D-TRYPTOPHAN ONLY, UNSTIRRED SOLUTION
CURVE 2. 5 x 10^{-3}M D-TRYPTOPHAN ONLY, STIRRED SOLUTION
CURVE 3. 1 ML D-AMINO OXIDASE ADDED TO 25 ML OF 5 x 10^{-3}M
          TRYPTOPHAN SOLUTION, UNSTIRRED SOLUTION
CURVE 4. 1 ML D-AMINO OXIDASE ADDED TO 25 ML OF 5 x 10^{-3}M
          TRYPTOPHAN SOLUTION, STIRRED SOLUTION
CURVE 5. 1 ML D-AMINO OXIDASE ADDED TO 25 ML OF 5 x 10^{-3}M
          TRYPTOPHAN SOLUTION, SOLUTION EXPOSED TO ATMOSPHERE, STIRRED SOLUTION

FIGURE 16. CHRONOPOTENTIOGRAM OF D-TRYPTOPHAN - D-AMINO ACID OXIDASE
            IN 0.1 M $Na_4P_2O_7$ BUFFER SOLUTION, 20 μ a

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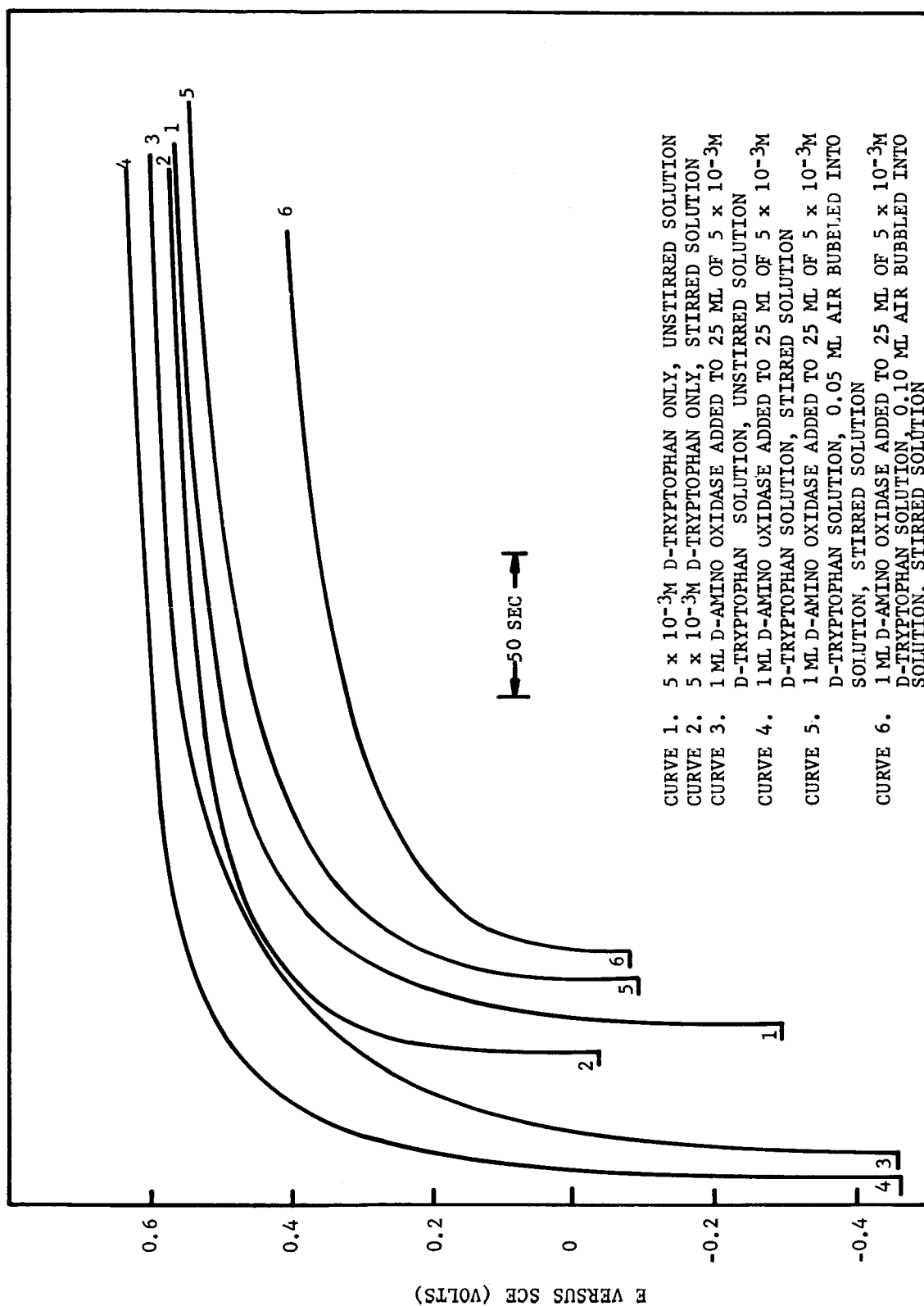
FIGURE 17. CHRONOPOTENTIOGRAM OF D-TRYPTOPHAN - D-AMINO ACID OXIDASE
IN 0.1 $Na_4P_2O_7$ BUFFER SOLUTION, 5 μ a

CURVE 1. 5 x 10^{-3}M D-TRYPTOPHAN ONLY, UNSTIRRED SOLUTION
CURVE 2. 5 x 10^{-3}M D-TRYPTOPHAN ONLY, STIRRED SOLUTION
CURVE 3. 1 ML D-AMINO OXIDASE ADDED TO 25 ML OF 5 x 10^{-3}M
          D-TRYPTOPHAN SOLUTION, UNSTIRRED SOLUTION
CURVE 4. 1 ML D-AMINO OXIDASE ADDED TO 25 ML OF 5 x 10^{-3}M
          D-TRYPTOPHAN SOLUTION, STIRRED SOLUTION
CURVE 5. 1 ML D-AMINO OXIDASE ADDED TO 25 ML OF 5 x 10^{-3}M
          D-TRYPTOPHAN SOLUTION, 0.05 ML AIR BUBELED INTO
          SOLUTION, STIRRED SOLUTION
CURVE 6. 1 ML D-AMINO OXIDASE ADDED TO 25 ML OF 5 x 10^{-3}M
          D-TRYPTOPHAN SOLUTION, 0.10 ML AIR BUBBLED INTO
          SOLUTION, STIRRED SOLUTION

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Some studies have been completed on the conditions required for separation of tryptophane, IPA and aerobic oxidation products of IPA electrophoretically.

In these studies the test material was absorbed onto 0.5 cm strips of Whatman 3MM paper and inserted into a similar area cut out from the strips soaked in buffer on the electrophoresis bed. Electrophoresis was continued at 500 volts during $2\frac{1}{2}$ hours and then the strips were cut into 1 cm pieces for analysis. The short cut pieces were placed in tubes containing 3 ml of 0.1 M pyrophosphate buffer at pH=8.3, shaken with a Vortex Mixer for 30 seconds and allowed to stand until time for spectrophotometric analysis. The solutions were filtered through a small glass wool plug directly into dry, 1 cm, silica cuvettes and examined in the region of 340 to 220 mu in the spectrophotometer. Materials compared were 1) tryptophane, 2) IPA, and 3) IPA in pyrophosphate buffer solution after having been allowed to stand one week in air. Runs were carried out at different pH values using buffers M/15 acetate, pH4.5 and M/15 phosphate, pH 7.0.

Tryptophan was mainly located in the first cm toward the negative pole in both buffers. The IPA, on the other hand appeared to be mainly in the 0.5 to 1.0 cm range on the positive side. However, IPA also showed other components, one moving as far as the 1.5 to 2.0 cm segment and the other in the same area as tryptophane. The fast moving IPA component had an absorption spectrum very similar to that of IPA but the other, the negative component, had a spectrum more akin to that of the oxidized solution of IPA.

Assay of the quantity of material associated with the separated fraction will be by radio-tracer methods. This involves the use of C^{14} labeled tryptophane as a substrate in the electrochemical cell. For this purpose the special cell, previously discussed and shown in Figure 1, will be used. It should permit the use of a minimum amount of radioactive material and give a maximum conversion to oxidized product per unit of volume.

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4.5 DISCUSSION OF RESULTS ON THE AMINO ACID OXIDASE SYSTEM

The foregoing results provide strong evidence that in the aerobic tryptophane-DAO system, the oxidation product indole pyruvic acid (IPA) is electrochemically active and is the species primarily responsible for the bio-electricity generated by this system. During the course of these studies however, observations accumulated, such as that mentioned in Section 4.1 and shown in Figure 6, which suggested that more than one form of IPA existed in solution and that these forms might not have the same electrochemical reactivity. Among such observations were: slow changes in absorption spectra during the period immediately after solution; inconsistencies between some of the curves found for IPA formed by DAO from tryptophan and those obtained for commercial IPA; rapid changes in current immediately following solution of IPA in the electrochemical cell; differences in currents found with freshly prepared and aged IPA solutions; a variable low voltage potential halt in chronopotentiometric measurements. Accordingly, a careful study of the probable species existing in solution was undertaken with spectrophotometric examination providing the main tool.

When IPA is dissolved in the usual buffer used in the electrochemical investigations of DAO action (pyrophosphate, 0.1 M, pH 8.3) and ultraviolet absorption spectra analyses made as soon as possible after addition of the solid IPA to the buffer, results such as those in Figure 18 may be demonstrated. Here, IPA (about 0.5 mg) was added, under a nitrogen atmosphere, to a thoroughly deaerated buffer (10 ml), dissolved rapidly and then transferred, still under nitrogen flushing, into the stoppered cuvette. As is shown, rapid changes in absorption occurred. In the first scan, at two minutes following addition of the solid to the buffer, high absorption is found for a peak at 306 μu but this decreases in a period of 40 minutes to a small shoulder on the side of a peak with maximum at 280 μu. Further decreases occur with time and the evidence shows clearly that less than 10 percent of the form which initially gave

1. 2 MINUTES AFTER SOLUTION
2. 8 MINUTES AFTER SOLUTION
3. 12 MINUTES AFTER SOLUTION
4. 20 MINUTES AFTER SOLUTION
5. 40 MINUTES AFTER SOLUTION
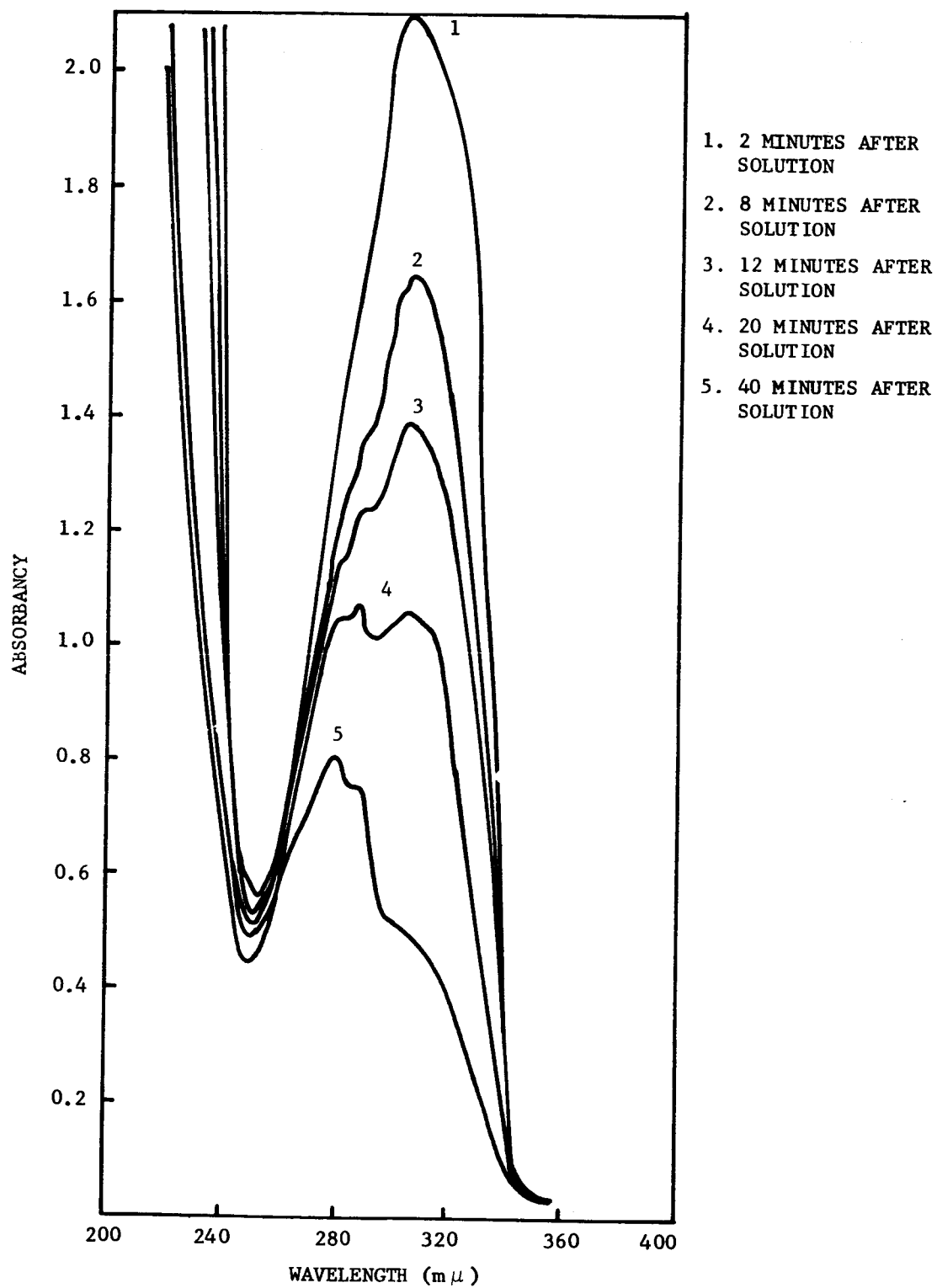
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FIGURE 18.   ABSORPTION SPECTRA OF IPA (ABOUT 0.05 MG/ML) IN pH 8.3 BUFFER, ANAEROBIC

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the highly absorbent peak is remaining at the later stages. Similar curves were obtained in water except that the changes did not occur as rapidly.

Figure 19 gives the UV spectra for IPA dissolved in methanol, acid and alkali. These curves were obtained by dissolving 24.2 mg of IPA in 10 ml anhydrous methanol, then adding 0.020 ml of this solution to 3 ml of the appropriate solvent in the cuvette, using a micrometer burette for measuring. In methanol little or no change occurred over a very long period of time, indicating that the form giving rise to the peak at 322 mμ is quite stable in methanol. In 1.0 M HCl, the peak is shifted to 327 mμ and diminishes very slowly with time, indicating that the acid catalyzes a change to a form which does not show this absorption. In NaOH, this peak disappeared except for a shoulder at 306 mμ but little or no change occurred in the peak at 272 mμ indicating stability in the chromophore giving rise to this absorption. Addition of sufficient acid to the alkaline solution caused partial regeneration of the long wave-length peak.

The IPA concentration versus cell current standardization curves also add evidence in favor of a change in IPA structure following solution in buffer. Currents obtained with freshly dissolved IPA did not follow the same linear curve found for predissolved, presumably equilibrated, IPA. This is shown by the two slopes exhibited by the curve of Figure 6, previously discussed in Section 4.1. Similarly, a change in structure could also be used to explain the rather steep initial drop in current found with IPA upon initial solution in the buffer of the cell. These results, found early in the investigation of IPA activity (Figure 20) were difficult to explain until the possibility of structural change was proposed.

The possible transformation of the IPA molecule which could lead to the observed behaviors, are 1) the keto-enol tautomerism, and 2) the acid-base equilibrium of the enol form. The former of these is believed most probably the one which explains the observed behavior, but data presently available do not exclude the latter. The keto-enol isomerization,

LIGHT PATH: 1 CM

A IN METHANOL
B IN 1 M HCl
C IN 0.01 M NaOH

FIGURES AT PEAKS
INDICATE LOG ϵ
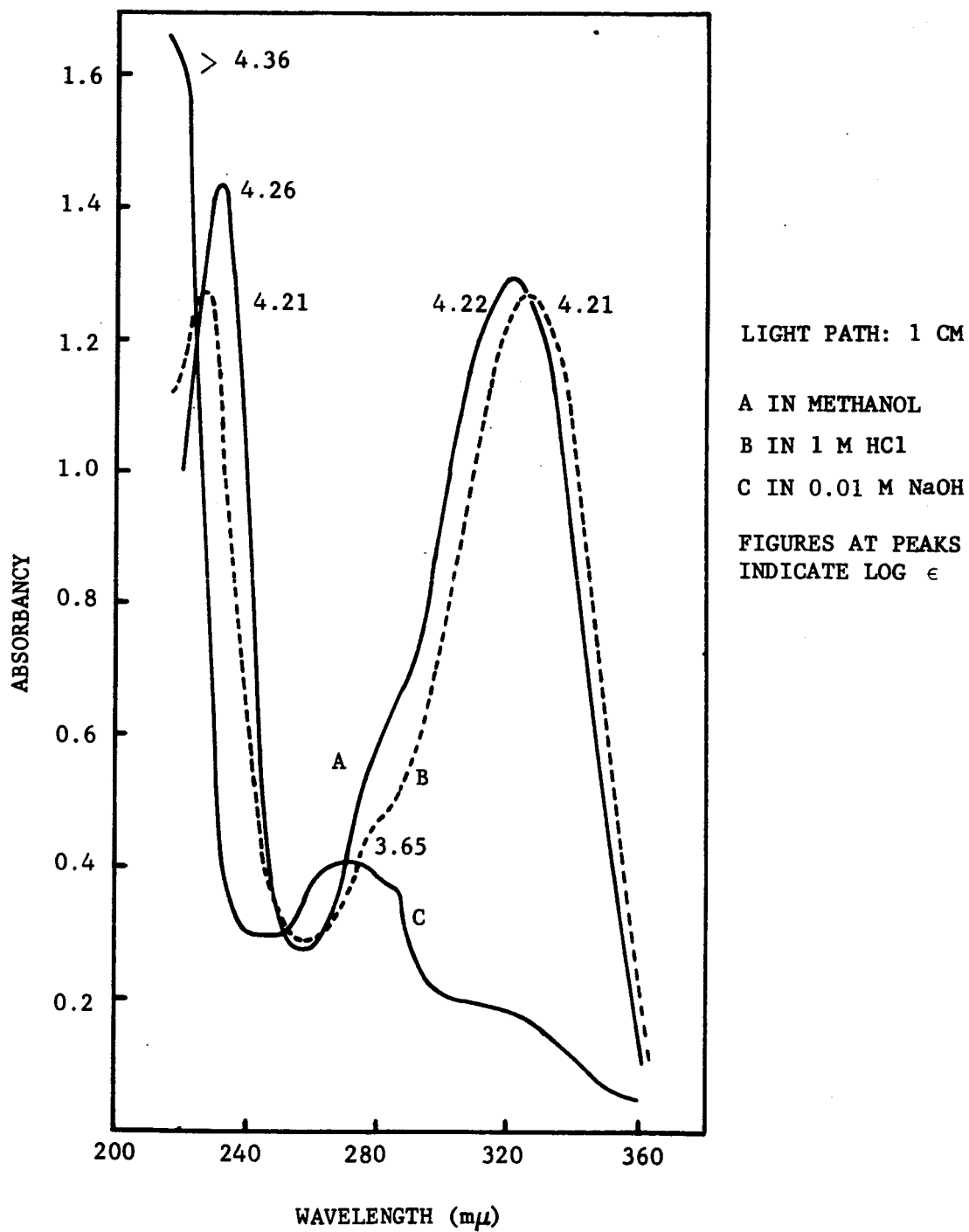
RO2656

FIGURE 19.   U-V SPECTRA OF INDOLE-3 PYRUVIC ACID $(7.9 \times 10^{-5} \text{ M})$
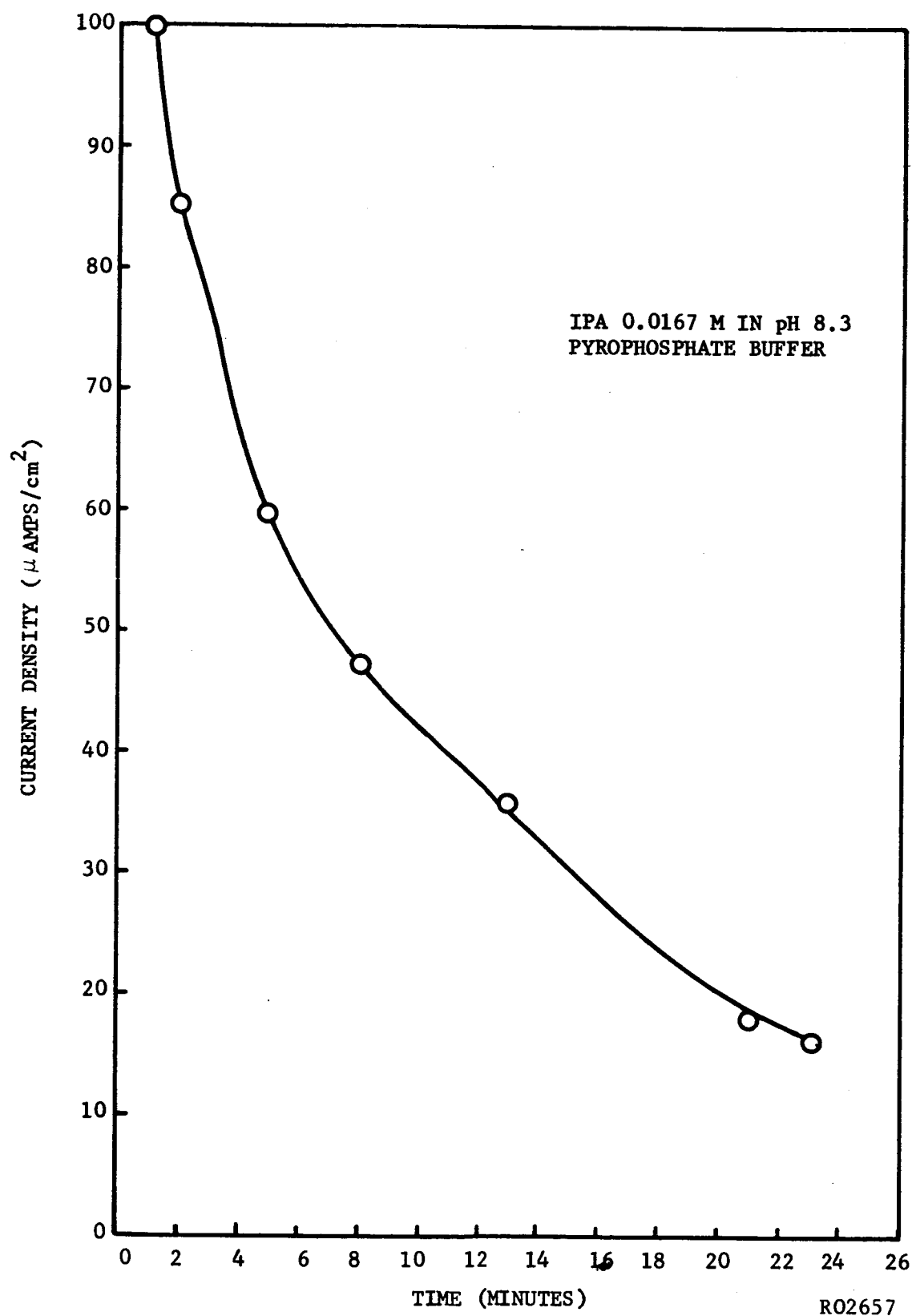IN VARIOUS SOLVENTS

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FIGURE 20. CHANGE IN CURRENT WITH TIME FOR FRESHLY DISSOLVED
IPA

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shown in Figure 21, would be acid and hydroxyl ion catalyzed. Structures, analogous to those of IPA, in which the stability of the enol form is greatly stabilized by formation of conjugated structures, are known to exist in large measure in the enol, rather than the keto form in solvents of low polarity or in liquid or gaseous state (7). In the case of IPA the conjugation of the unsaturated ring with the enol double bond and the carbonyl of the carboxyl group would be expected to cause such stabilization. Thus, even in aqueous solution, a significant concentration of the enol form is likely to be present at equilibrium. In basic solution, conversion to the enolate (salt form) is a possible explanation for disappearance of the enol peak at 322 mμ.

Spectrophotometric studies of keto-enol tautomerism with other materials have revealed spectral shifts similar to those observed here. For example, ethyl acetoacetate in the keto form shows only a weak absorption band in the near UV at 275 mμ, (molar absorptivity, E, = 16) characteristic of the weak R-band absorption of the isolated keto group. The enol, however, shows a K-band shift to give a strong absorption at 244 mμ, (E = 16000) (8), which may be ascribed to augmented absorption from the conjugation between the carbony of the ester group and the enol double bond.

Similarly, for the keto form of cyclohexane-1,3-dione, a weak absorption at 280 mμ (E=30) is found while for the enol form the maximum at 255 mμ is very strong (E=16000). (9)

In IPA, the conjugated structure in the enol should give rise to a rather strong absorption in the longer wavelengths. A comparison of the spectral characteristics of IPA in methanol solution with other 3-substituted indoles is given in Table 4. This illustrates the highly effective conjugated structure which must be present in order to shift the maximal wavelength so far into the long wavelengths. Indole carboxaldehyde has a more significant shift to longer wavelengths than that found in indole acetaldehyde, probably because of the direct conjugation of the
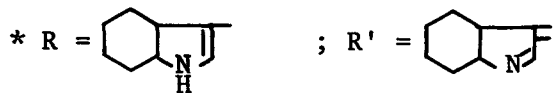
-44-
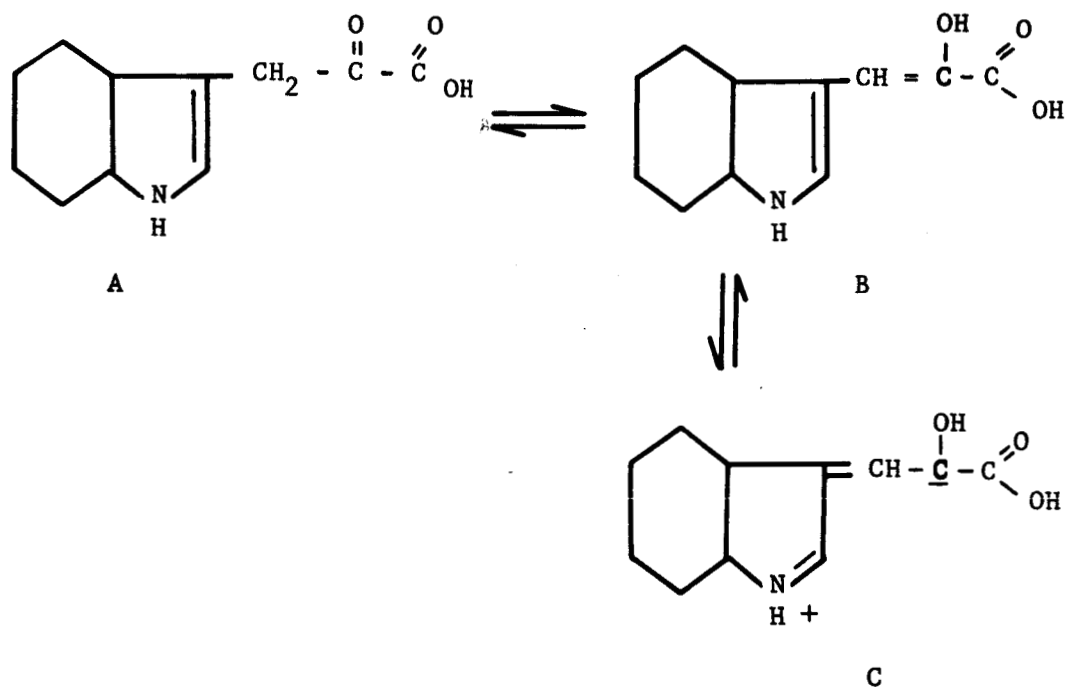
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FIGURE 21.   KETO-ENOL TAUTOMERISM OF IPA

TABLE 4

SPECTRAL CHARACTERISTICS OF INDOLE DERIVATIVES RELATED TO IPA

Compound[*]	Solvent	λ max[**] Log E					
Indole R-H	EtOH	222 4.50		265 3.82	280 3.75	289 3.65	
3(2-aminoethyl)-indole R-CH$_2$-CH$_2$NH$_2$	EtOH	222 4.50		276 3.70	282 3.72	291 3.65	
3-allyl indole R-CH=CH$_2$	EtOH	220 4.54		274 3.8		286 3.72	
Indole-3-carbox-aldehyde	EtOH		243 4.07	260 4.00			296 4.07
R-CHO	H$_2$O	210 4.4	245 4.1	260 4.1			300 4.1
	NaOH			263 4.2			323 4.1
Indole-3-acet-aldehyde R-CH$_2$-CHO	EtOH	222 4.51			280 3.78	289 3.70	
Indole-3-pyruvate R-CH$_2$-CO-COOH	MeOH	227 4.26		280 ?			322 4.22
3-(dimethylamino)-methylene indolinene R'=CH-N(CH$_3$)$_2$	MeOH acid	215 4.50	248 4.1				338 4.25
3-(methylamino)-methylene indolinene	Organic	220 4.4		260 4.25		285 4.1	
R'=CH-NHCH$_3$ acid		210 4.45		265 4.1			325 4.25

* R = (indole ring structure, N-H) ; R' = (indolenine ring structure, N)

** E = extinction coefficient

carbonyl group with the ring unsaturation. A part of this shift must be due to added structural variations, however, since 3-allyl indole does not show the same degree of shift found for the carboxaldehyde. In all probability, the keto-enol tautomerism is not the only effect involved in the IPA transition since the presence of potential ionizing groups such as the carboxyl and secondary amine group would have a profound influence on the structures under ionizing conditions. Thus, the possibility of charge separation structures, giving double bond conjugation at the nitrogen and the 3-methylene group should give high absorptions at long wave lengths, as shown by the indolinene derivatives in the table.

On the basis of the foregoing considerations it is concluded that the species responsible for electrochemical activity in the tryptophane-DAO system is the enol form of indole pyruvic acid. The chronopotentiometric curves indicate that this species is readily oxidized at a potential of about 0-0.2 volts vs SCE, thereby having the capability of providing a potential of about 0.6-0.8 volts vs an oxygen electrode in a practical bio fuel cell. Unfortunately this species does not predominate at equilibrium and the preponderate keto form is apparently no more readily oxidized than tryptophane itself.

These conclusions mean that the currents obtained from this system in the electrochemical cell at a potential of 0.2 volts are due to the small equilibrium concentration of this enol form, and make it clear why this current, as it was found to be, is strongly dependent upon stirring rate. It is not known whether the keto or the enol form of IPA is first formed as a result of the enzyme action on tryptophane. If the latter, however, then it is clear that advantage is to be gained from a cell configuration which provides maximum opportunity for the IPA to be oxidized at the electrode surface directly as it is formed. Such a configuration is clearly that in which the biological agent is confined in the vicinity of the electrode surface. It is quite possible that a

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situation such as this is responsible for reported observation of improved performance by bioelectrodes in which the bacterial agent was coated onto the electrode surface.

It is believed that considerable importance should be attached to the observations, discussed in the foregoing, that electrode surfaces can be poisoned by the oxidation produced from biological systems and become inactive for further generation of bio-electricity. The results show that once such poisoning has occurred, the electrode can be reacti- vated either by chemical cleaning or by prolong electrochemical reduction. This fact may be responsible for some of the disparity between observa- tions on bio-electrochemical activity reported by various workers in this field.

SECTION 5

CHARACTERIZATION OF THE UREA-UREASE SYSTEM

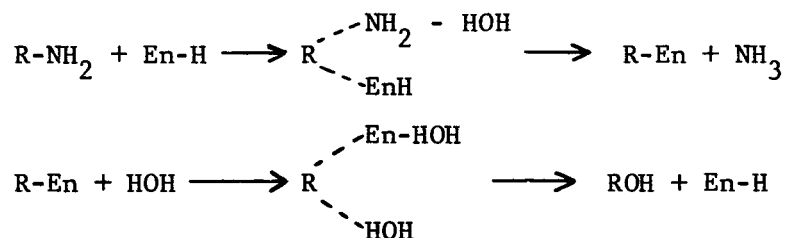The action of urease on urea consists solely of hydrolysis according to the following general reaction:

$$\begin{array}{c} NH_2 \\ \diagdown \\ \diagup \\ NH_2 \end{array} C=0 + H_2O \xrightarrow{\text{urease}} 2NH_3 + CO_2$$
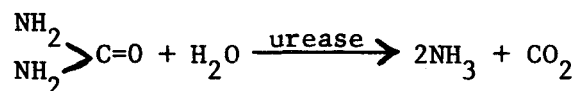
Although this is accompanied by a substantial energy release, it is difficult to envision how it can be harnessed to an electrode process.

The detailed mechanism of the hydrolysis has not been entirely settled. However, it is probably a two step process, going through a series of bond exchanges of the type

$$R-NH_2 + En-H \longrightarrow R \begin{array}{c} \diagup NH_2 - HOH \\ \diagdown EnH \end{array} \longrightarrow R-En + NH_3$$

$$R-En + HOH \longrightarrow R \begin{array}{c} \diagup En-HOH \\ \diagdown HOH \end{array} \longrightarrow ROH + En-H$$
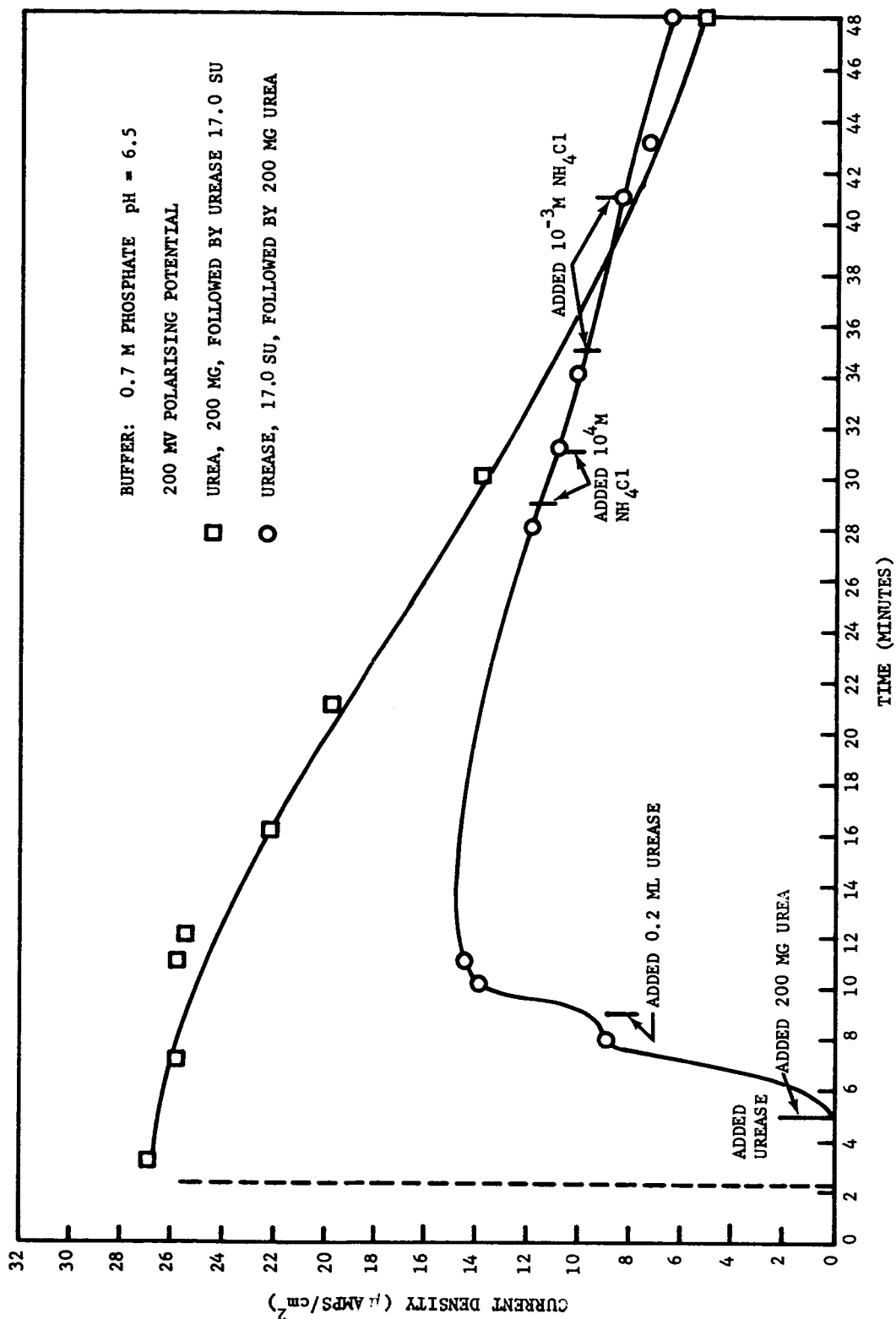
In which all except the final step, splitting of the enzyme-substrate intermediate are highly reversible. This process goes through the formation of an intermediate carbamide (10) which is subsequently hydrolysed to the final products of NH_3 and CO_2. It follows that for electrochemical

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activity to be possible one or more of these intermediate or final reaction products must undergo oxidation at the electrode. It would be of considerable interest to determine if this actually does occur, and if so, which species are involved.

Early studies on the urea-urease system at Aeronutronic indicated that it possessed significant electrical activity which could not be attributed to either the urea or the urease alone. This is indicated by the two curves of Figure 22 which show the effect of adding, in the one case urea to a urease solution in an electrochemical cell, and in the other, addition of urease to a urea solution. It is noted that in both cases, current is negligible until the second constituent is added, after which it rises rapidly to a peak and then decreases at a gradual rate over a fifty minute period of observation. These currents were obtained at the usual electrode potential of 0.2 volts vs the SCE and in a phosphate buffer at pH of 6.5.

In subsequent investigations, of a similar nature, significant currents were not obtained at the potential of 0.2 v. but were obtained at 0.6 v and in a buffer made up of tris (hydroxymethyl) amino methane having a pH of 8.0. In this case, a current was obtained from both the enzyme and urea alone, but significantly greater values were obtained when both were present together. The increase appeared to derive from the hydrolytic reaction between urease and urea. These observations are shown on Figure 23.

The enzyme used in the foregoing observations was a crystalline preparation having a very high activity of 11000 to 13000 SU per gm. When similar observations were made using a commercial urease preparation having a much lower activity 350 SU per gm., results were obtained as shown in Figure 24. Here a very high background current was obtained from the enzyme alone, but a significant increase was again obtained upon addition of urea. Use of a sample of this enzyme purified by dialysis gave a slightly lower background current but a similar incremental increase

FIGURE 22. ELECTROCHEMICAL ACTIVITY OF UREASE WITH UREA

RO2659

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VOLTAGE = +0.600
BUFFER = 0.1 M TRIS PH 8.0

DIALYZED UREASE, 129 SU, FOLLOWED BY 450 MG UREA

450 MG UREA FOLLOWED BY UREASE

ADD UREASE

ADD UREA

TIME (MINUTES)

CURRENT DENSITY (μAMPS/CM²)

RO2660
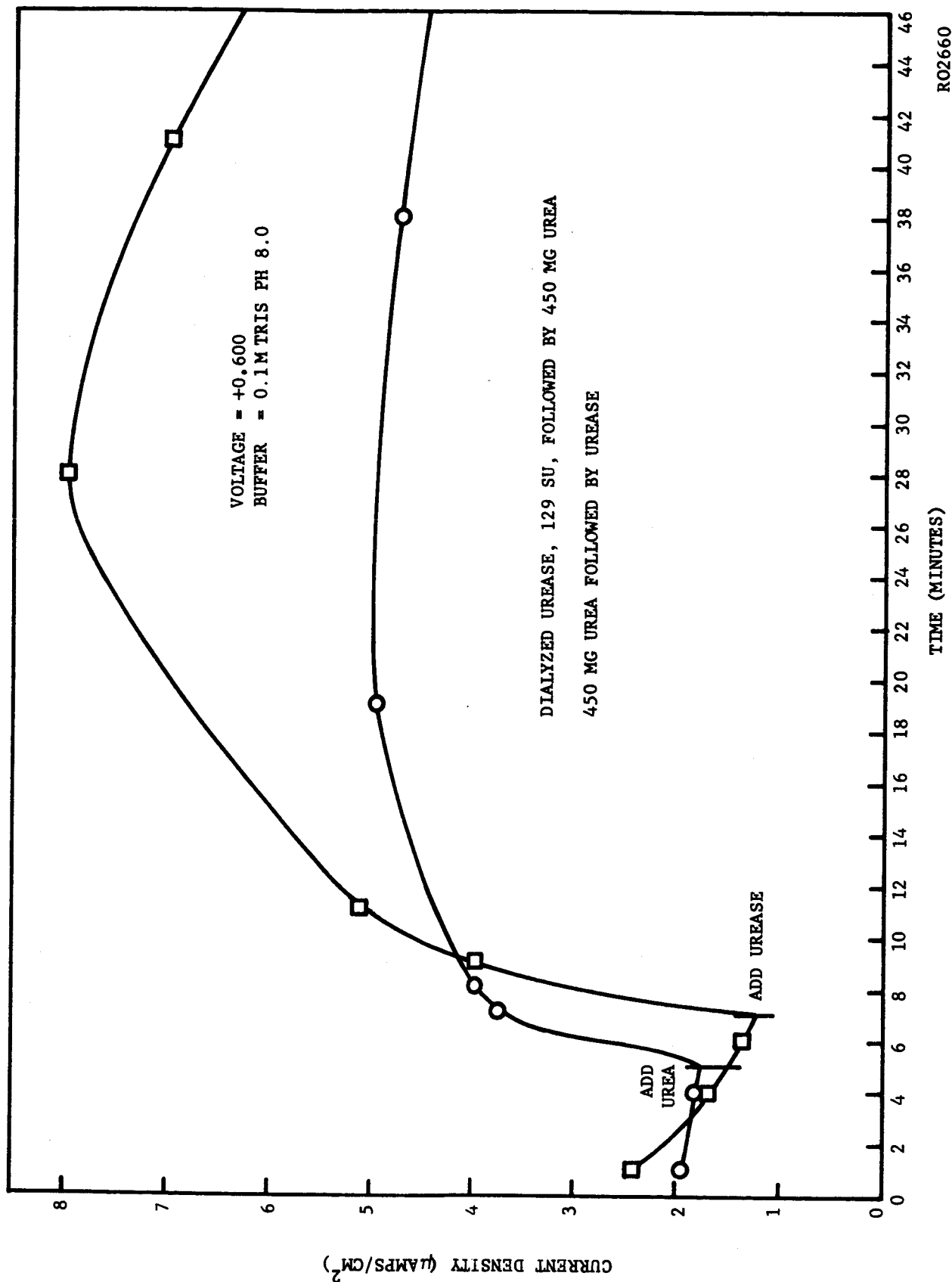
FIGURE 23.   ELECTROCHEMICAL ACTIVITY OF UREASE IN UREA

-52-
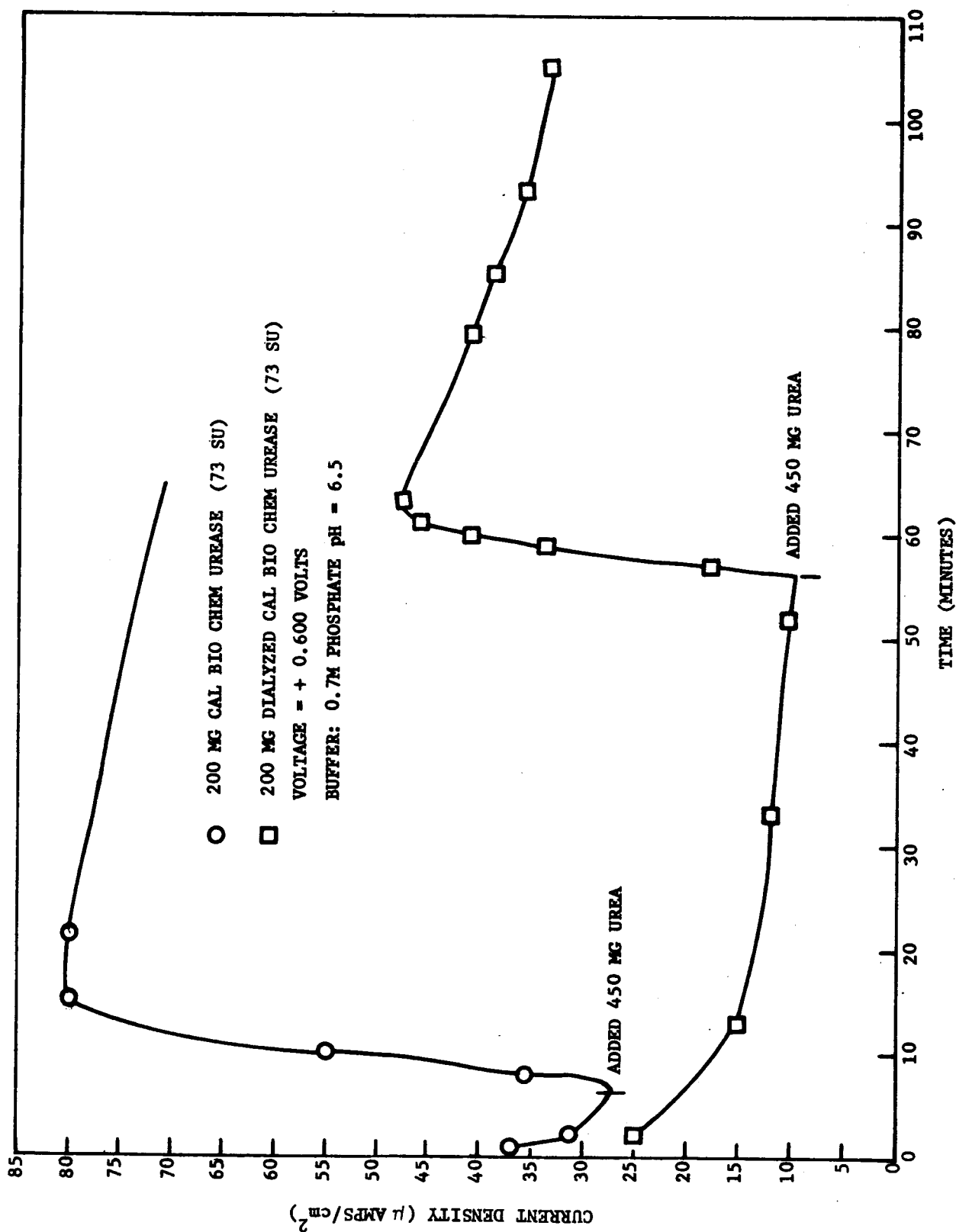
FIGURE 24. ELECTROCHEMICAL ACTIVITY OF COMMERCIAL UREASE - UREA SYSTEM

O 200 MG CAL BIO CHEM UREASE (73 SU)

□ 200 MG DIALYZED CAL BIO CHEM UREASE (73 SU)

VOLTAGE = + 0.600 VOLTS

BUFFER: 0.7M PHOSPHATE pH = 6.5

ADDED 450 MG UREA

ADDED 450 MG UREA

CURRENT DENSITY (μ AMPS/cm²)

TIME (MINUTES)

RO2661

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upon addition of urea. The effect of pH on current obtained from the commercial enzyme in the absence of urea is shown by the curves of Figure 25 obtained with two different buffers at pH of 6.5 and 8.0. The seven fold increase in initial current obtained upon changing pH from the lower to the higher value appears to be quite striking.

No satisfactory interpretation of these observations is presently available and the urea-urease system remains somewhat of an enigma. Seemingly, in a reaction system involving no formal oxidation-reduction processes, it is possible to obtain an electrochemical reaction related in some way to the hydrolytic process. The explanation of the effect most commonly advanced is that ammonia formed in the hydrolysis in the electroactive species. However ammonia is not known to be readily oxidized at an electrode. Its behavior in the urea-urease system is shown on Figure 22 where it is added to the system as NH_4Cl at the two points indicated with no measurable effect on the electrical output.

Further study of the urea-urease system is planned with particular attention to the possible role of impurities in the enzyme preparation.
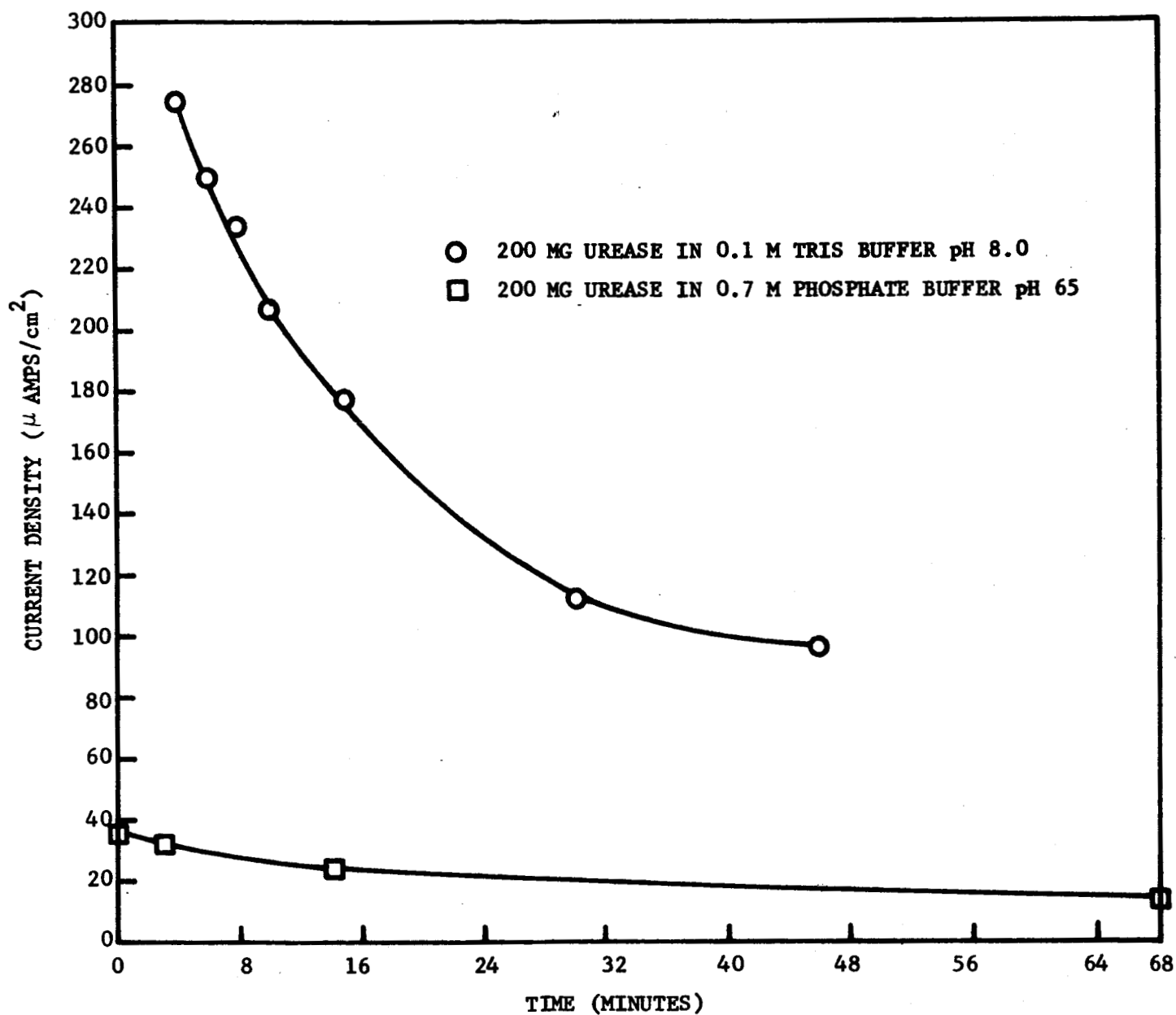
FIGURE 25. EFFECT OF BUFFER AND pH ON CURRENT FROM UREASE

RO2662

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SECTION 6

ELECTRODE COATING STUDIES

6.1 EXPERIMENTAL STUDIES

The potential value of having organisms attached to or incor-
porated in the electrodes must arise from one or more of these possible
effects: 1) shorter diffusion paths between the electrode and the bio-
logical reactive site; 2) possible direct interaction between the
organized structure of the cell and the electrode, either in providing
a direct electron transport from cell interior to the electrode or in
direct reaction of an enzyme with the electrode surface; 3) potential
effects on a bacterial cell or enzyme arising from the high potential
gradients present in the electrical double layer. The possibility of
directed diffusional flow of substrate or reaction intermediates through
the biological system as a consequence of such gradients has been sug-
gested.

The kinetics of the first case may be studied with well
characterized enzyme systems by varying the parameters of enzyme and
substrate concentration as well as distance of the reaction from the
electrode. This may be accomplished, to a limited degree, by enclosing,
or imbedding, the enzyme in a semipermeable material in the vicinity of
the electrode.

In the second case the question of direct reaction of the enzyme

at the electrode would initially involve a search for an enzyme which
actually could be demonstrated to react at the electrode, and determina-
tion of the conditions which would promote such reaction. To a consider-
able extent, the work described in this report on the amino acid oxidase-
amino acid system deals with this problem. The question of electron con-
duction from within a bacterial cell is somewhat more difficult concep-
tually, but would probably involve demonstration that physical contact
between the cell and an electrode surface has an important bearing on
electrode current.

The third effect has not been subjected to extensive study as
yet. Here, the use of model enzyme or cell systems is a possibility.
Potential differences could be created with the use of double membranes,
which provide an enzyme or cell enclosure, and the potentials of which
could be individually controlled. Lipoprotein emulsions or membranes
might lend themselves to such a study of the potential gradient problem.

Present endeavors, summarized below, have been mainly limited
to exploration of the first effect indicated above, namely the question
of proximity to the electrode in terms of diffusion pathlength from the
biological unit to the electrode surface.

A first approximation to an enzyme on the electrode surface
can be achieved by the incorporation of the enzyme into a fixed, permeable
matrix on the surface. Initial experiments in this connection were per-
formed by simply incorporating the enzyme solution into warm agar-buffer
mixture, coating the flat electrode with a thin layer of the liquid agar
by dipping and allowing it to cool and harden after removing the excess
droplet. Although such experiments with the normal flat electrode were
reasonably successful, the adherence of the agar layer was rather irregular
and it would frequently become partially detached during an experiment.
Consequently, a modified electrode was devised consisting of a spiral of
0.050" platinum wire. This had a total surface of 3.54 cm (calculated) in
place of the 4.0 cm area of the flat electrode. Tests of currents obtained

with the DAO system indicated that the spiral electrode was equivalent to the flat electrode in current collection except that some differences were noted under discontinuous operation. With the flat electrodes, a normal small current surge generally occurred immediately following the resumption of the discharge cycle after a rest period. With the spiral electrode, this transient was greatly increased, currents double the equilibrium value being obtained under some circumstances.

In Table 5 are reported some data obtained on the effect of agar coating with this electrode (similar results were obtained with the flat electrode). When the enzyme is present only in the agar coating but not in the free solution (Case A) the enzyme concentration in the region of the electrode, is approximately seven times greater than that normally used with the enzyme in free solution but only about one-hundredth the normal total amount of enzyme is used. As shown in the table, the coated electrode in Case A did not support any current under anaerobic conditions but a small, stable current could be obtained in the presence of air. It is also seen that the low current is associated with a low IPA value found in the surrounding medium. When the enzyme is present in the medium surrounding the agar coated electrode, but not in the agar (Case B) it was possible to obtain a current under both conditions, aerobic as well as anaerobic and a much higher concentration of IPA is found in the free solution. Removal of the agar, however, (Case C) revealed that this concentration of IPA in solution was sufficient to support a much greater current on the bare electrode than was obtained with the coated electrode.

In the above experiment, it is uncertain as to how much of the current reduction in the presence of agar is due to simple interference of diffusion of IPA through the agar and how much might be due to other problems, such as making the collector surface unavailable by the coating or interfering with electrode reaction mechanisms. To test the diffusion properties of the system, an electrode coated with agar containing no

TABLE 5

ENZYME INCORPORATION IN AGAR COATINGS ON ELECTRODES

Conditions:

A: Enzyme in agar. 0.5 ml DAO, 0.02 ml catalase, 0.5 ml P-P buffer in 1 ml 2% agar, 17 mg. of mixture (equivalent of 0.005 ml DAO) coated on coil electrode (3.54 cm total surface) Tryptophane, 0.005M, in P-P, pH 8.3 buffer.

B: Enzyme in free solution, agar coated electrode. Electrode coated with 17 mg agar mixture as above with buffer replacing enzyme. 0.5 ml DAO, 0.1 ml catalase in tryptophan-buffer solution used above.

Condition	Atmosphere	$V_o(mv)$*	$V_c(mv)$*	$I_c(\mu a/cm^2)$*	IPA** $Mx10^3$
A. Enzyme in Agar	N_2	90	Unstable	Unstable	0.05
	Air	85	240-290	2.5	0.1
B. Enzyme in Solution Electrode Agar Coated	N_2	110	200	2.7	0.1
	Air	-	200	6 — 20	1.4
C. Electrode Bare	N_2	-	200	55	1.5

* V_o - open circuit potential

  V_c - closed circuit potential

  I_c - load current

** Apparent indole-3-pyruvic acid concentration determined spectrophotometrically

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enzyme was tested for current production by IPA-tryptophan mixtures in the presence and absence of enzymes. In Figure 26 is shown the results under intermittent load cycles. The procedure involved first loading the electrode at the regular potential of 0.200 v vs SCE. After the current decayed partially, the circuit was opened and the electrode allowed to recover for a few minutes and then the load reimposed. As the curves show, there was a significant recovery in current in each case during the rest period. These results suggest at first that the IPA concentration in the region accessible to the electrode was being reduced during the work cycle more rapidly than it could be replenished by diffusion through the agar. However, it was subsequently found that removal of the agar did not alter the decay-recovery pattern and the currents found were quite similar whether agar was present or not. The observed effects were quite reproducible and the recovery seems to be a property of the electrode rather than of the agar coating. The agar seems to be essentially transparent to the IPA.

Since the use of an agar matrix for imbedding an enzyme involves possible partial denaturation of the enzyme and also may restrict approach of the substrate, it was thought desirable to consider a controlled system in which the enzyme is free to orient itself within a limited space. The first approximation to this is the use of semipermeable dialyzing membranes to hold the enzyme in the region of the electrode (or, conversely, a short distance away from the electrode) while allowing free diffusion of the substrate and oxidizable material. Early trials with such an arrangement, using DAO and tryptophane, were unsuccessful. Rapid polarization of the electrode occurred under all circumstances because of lack of stirring within the membrane-electrode area.

Further work on this aspect of the problem will be carried out using a specially fabricated electrode chamber wherein the membrane to electrode distance may be varied at will and stirring of the electrode compartment solution may be accomplished by use of a vibrating electrode.
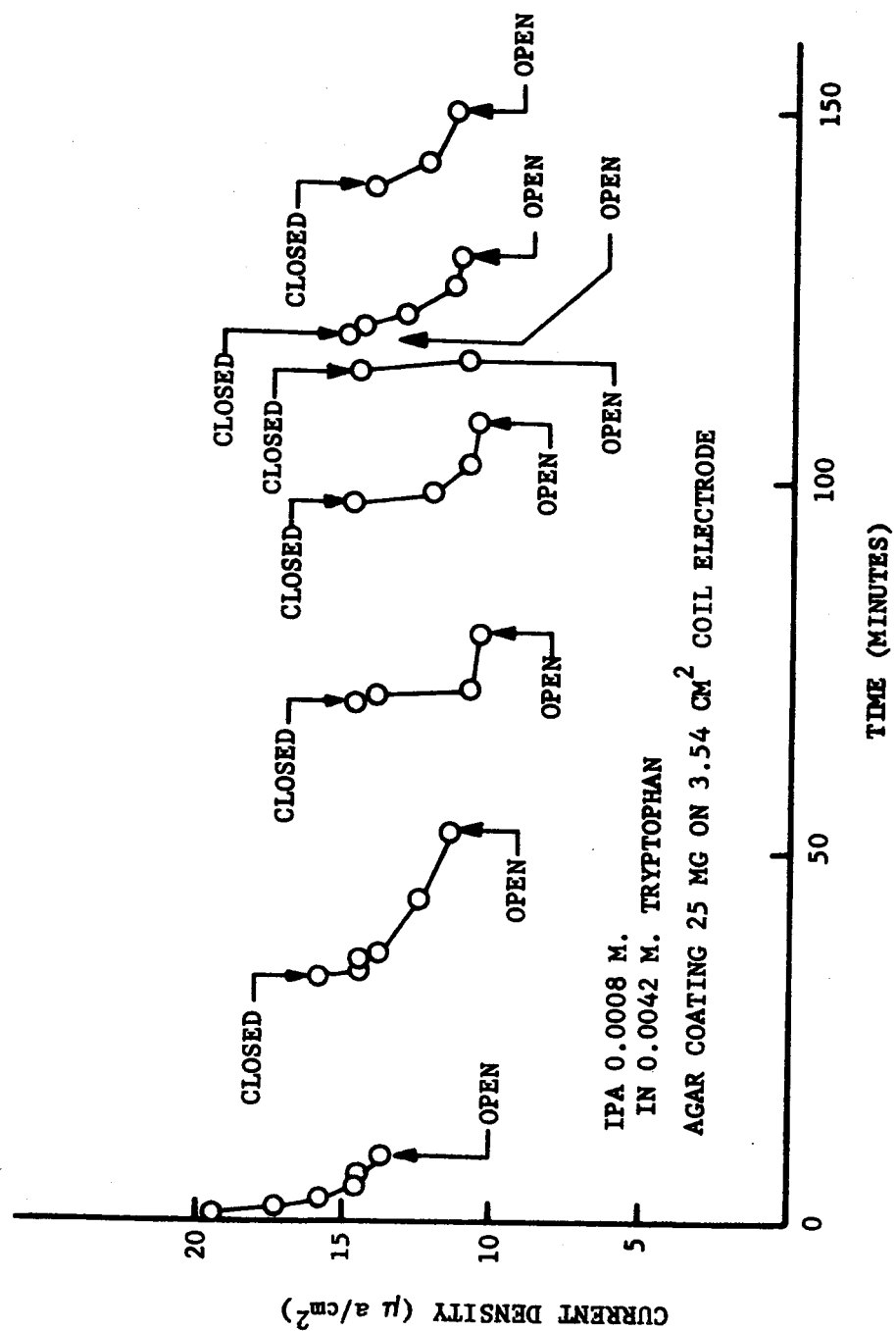
FIGURE 26. EFFECT OF INTERMITTENT RECOVERY PERIODS ON CURRENT FROM IPA WITH AGAR COATED ELECTRODE

RO2663

IPA 0.0008 M.
IN 0.0042 M. TRYPTOPHAN

AGAR COATING 25 MG ON 3.54 CM^2 COIL ELECTRODE

TIME (MINUTES)

CURRENT DENSITY (μ a/cm^2)

6.2 DISCUSSION

The enzyme coating studies are somewhat difficult to assess at this point. The data indicate that the agar, itself, does not interfere significantly, with the oxidation of the IPA present in free solution. However, incorporation of enzyme into the agar does not allow current values which approach those obtained with the enzyme in the free solution. Also, with the enzyme in free solution, there is a significant reduction in current when the plain agar (without enzyme) is used as an electrode coating vs the uncoated electrode, even though the concentration of IPA in solution is the same in both cases. These observations are presently inexplicable. Further support for some sort of enzyme-electrode inter-action may be taken from the data in Table 2, in which electrode currents are shown to be greater at a given IPA concentration, with enzyme present than without the enzyme. Examination of this question will continue.

FUTURE WORK

1. Electrode attachment and incorporation studies will continue with the use of DAO (as the only completely characterized enzyme system presently available for model systems) incorporated into different media or directly adsorbed to the electrode surface. A special anode compartment will be used allowing more versatility in providing varied enzyme and substrate concentrations in different positions with respect to the electrode. Of special interest will be the problem of differential potential gradients across membranes and their effects upon substrate and product flows.

2. Studies will be carried out upon intact micro-organisms and the effect of their attachment to the electrodes. They will include investigation of the alteration in conductive properties of the electrode system by bacterial coatings and attempts will be made to relate observed potential and current effects with theoretical parameters.

3. Urease-urea systems will be further characterized so as to determine the cause of observed electrical activity and to determine conditions for optimal production of electrical energy by this system.

4. Work on characterization of the general mechanism by which biological systems produce electrical energy will be continued, with continued interest in the question of direct enzyme or macro molecule participation in the electrode reaction. Selection of additional suitable organism or enzyme candidates for study are presently in progress. Those associated with nitrogen metabolism are presently favored.

5. The chronopotentiometric studies on the selected biological systems will continue. Work in the near future will be directed to the evaluation of wax impregnated carbon electrodes for this purpose.

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